The Aryl Hydrocarbon Receptor and the Cardiovascular System in Zebrafish (*Danio rerio*)

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ABSTRACT

Developmental exposure to aryl hydrocarbon receptor (AhR) agonists in fish causes severe defects in the cardiovascular system. However, the effects of acute AhR agonist exposure on the adult fish cardiovascular system as well as the genes mediating developmental AhR-induced deformities remain unclear. In this thesis, two studies were carried out to address these issues. Before experiments could begin, methods for quantitative real-time reverse transcriptase polymerase chain reaction (rtrt-PCR) as well as larval exposure and rearing were developed, validated, and optimized.

Following method development, a series of experiments was performed on adult zebrafish (*Danio rerio*) to assess how expression of cytochrome P450 (CYP) and cyclooxygenase (COX) enzyme mRNA in hepatic and vascular tissues is altered after intraperitoneal injection of AhR agonists benzo(a)pyrene (BaP) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alone and in combination with the purported AhR antagonists resveratrol (Res) or α-naphthoflavone (ANF). Both TCDD and BaP induced similar patterns of gene expression in arteries, although with different efficacies, and had slightly different effects in hepatic tissues. Resveratrol was generally without effect in all treatment groups and tissues with the exception of reducing TCDD-induced CYP1C2 in vascular tissues. In contrast, ANF antagonized TCDD- and BaP-induced changes, as well as reduced baseline gene expression in liver. However, in arteries, ANF alone acted as an agonist to increase expression of several of the genes investigated.

The second series of experiments involved zebrafish eggs aqueously exposed to BaP or TCDD alone and in combination with Res or ANF. Whole larvae CYP and COX isoform mRNA expression was quantified at 5 and 10 days post-fertilization (dpf), then correlated with developmental phenotype. Both TCDD and BaP caused concentration-dependent AhR-associated deformities with a significant increase in mortalities by 10 dpf and increased CYP1A mRNA expression, while TCDD alone decreased CYP1C2 expression. BaP/ANF co-exposure exhibited the highest rate of deformities and mortalities at both 5 and 10 dpf, caused marked alterations in cardiac and vascular morphology at 10 dpf, and increased CYP1A expression. Furthermore, ANF exhibited additive agonistic effects on gene expression with both BaP and TCDD. Correlation analyses revealed that

gene expression at 5 dpf, but not 10 dpf, was strongly linked to abnormal cardiac and vascular phenotypes at 10 dpf with several genes related to cardiac development and one primary gene linked to vascular development.

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A – atria

ACh – acetylcholine

AhR – aryl hydrocarbon receptor

AHR – gene encoding for the aryl hydrocarbon receptor

AIP – aryl hydrocarbon interacting protein

ANF – α-naphthoflavone

ANOVA – analysis of variance

ARNT – aryl hydrocarbon receptor nuclear translocator

BaP – benzo(a)pyrene

bp – basepairs

bpm – beats per minute

°C – degrees Celsius

cAMP – cyclic adenosine monophosphate

CCV – common cardinal vein

cDNA – complementary deoxyribonucleic acid

CFM – craniofacial malformations

Con - control

COX - cyclooxygenase

ΔCSA – change in cross-sectional area

ΔΔCt – change in threshold values; used in real-time reverse transcriptase PCR

CYP – cytochrome P450 monooxygenase

DA – dorsal aorta

dia - diastolic

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

dpf – days post-fertilization

DRE – dioxin response elements

eNOS – endothelial nitric oxide synthase

EP – receptor for prostaglandin E2

ER – endoplasmic reticulum

EROD – ethoxyresorufin-O-deethylase

g – gram

G3PDH – glyceraldehyde-3-phosphate dehydrogenase

h – hour

H – heart

HAH – halogenated aromatic hydrocarbon

hpf – hours post-fertilization

HSP90 – heat shock protein (90 kiloDaltons)

iNOS – inducible nitric oxide synthase

ip – intraperitoneal injection

IP3 – inositol triphosphate

kg - kilogram

L - liter

L-NAME – N-nitro-L-arginine methyl ester

M – molar; moles per liter

mg – milligram

ml – milliliter

mm – millimeter

mM – millimolar

mRNA – messenger ribonucleic acid

MS-222 – tricaine methanesulfonate

n – number of individuals in a sample (number of replicates)

NE – norepinephrine

ng – nanogram

nNOS – neuronal nitric oxide synthase

NRF2 – nuclear erythroid 2 p45-related factor 2

NSAID – nonsteroidal anti-inflammatory

NTC – no template control

p – p-value

PAH – polycyclic aromatic hydrocarbon

PAS – Per/ARNT/Sim

PCA – principle components analysis

PCB-126 – 3,3',4,4',5-pentachlorobiphenyl

PCE – pericardial edema

PCR – polymerase chain reaction

PCV – posterior cardinal vein

pg – pictogram

PG - prostaglandin

PGI2 – prostacyclin

r – Pearson correlation coefficient

Res – resveratrol

RNA – ribonucleic acid

rtrt-PCR – real-time reverse transcriptase polymerase chain reaction

SC – spinal curvature

SEM – standard error of the mean

SNP – sodium nitroprusside

sys – systolic

TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

TGF-β – transforming growth factor β

TxA2 – thromboxane

μg – microgram

μl – microliter

μm – micrometer

V - ventricle

v/v – volume per volume of solvent to diluent

WL – whole larvae

w/v – weight of solute per volume of solvent

XAP-2 – hepatitis B virus X-associated protein

YSE – yolk sac edema

1.0 INTRODUCTION

1.1 Main Theme of Thesis

The major theme of this thesis is an exploration of the mechanisms of cardiovascular toxicity in fish produced by the common environmental contaminants, benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. After developing several methods needed for the main work of the thesis, the first study of this thesis examines effects of these contaminants when adult fish are acutely exposed. This is followed by another series of experiments examining effects of these contaminants after developmental exposure in fish.

1.2 Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is a member of the PAS (Per/ARNT/ Sim) protein family which have a basic helix-loop-helix conformation. While an endogenous antagonist, 7-ketocholesterol, has been found, there is no confirmed endogenous agonist, although several ligands are candidates including some arachidonic acid metabolites (Karchner et al., 1999; Andreasen et al., 2002a; Hahn, 2002; Nguyen and Bradfield, 2008). The AhR is a ligand-dependent cytosolic transcription factor which functions in mediating toxicological responses to various environmental contaminants, such as dioxins, benzo(a)pyrene, and polychlorinated biphenyls, which bind to AhR with high affinity (Andreasen et al., 2002a; Hahn, 2002; Denison and Nagy, 2003), while its role in normal development remains unclear. In its non-liganded bound form, the AhR resides in the cytosol of a cell, complexed with two molecules of heat shock protein 90 (HSP90) and with an aryl hydrocarbon interacting protein (AIP), also known as the hepatitis B virus X-associated protein (XAP-2) in mammals (Figure 1.1). This complexation allows the AhR

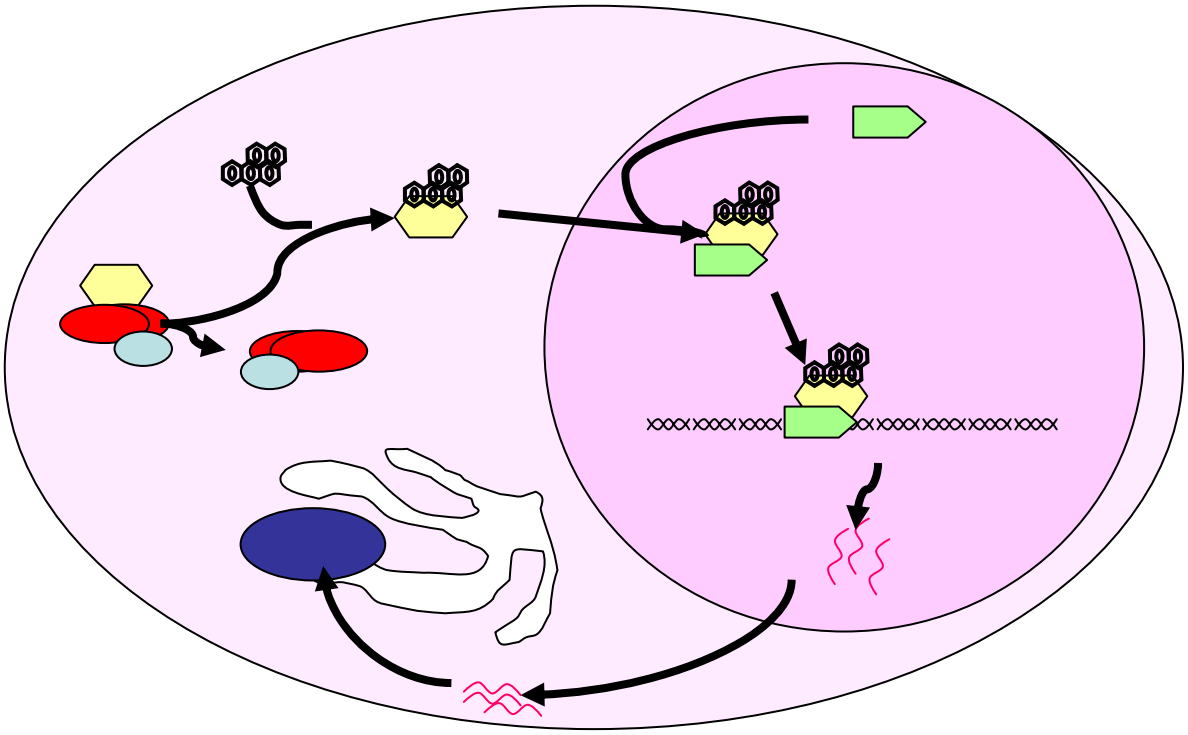


Figure 1.1: Simplified schematic of the aryl hydrocarbon receptor mechanism of enzyme induction. AHR – Aryl hydrocarbon receptor; HSP90 – heat shock protein; AIP – aryl hydrocarbon interacting protein; ARNT – AHR nuclear translocator; ER – endoplasmic reticulum; CYP1A – cytochrome P4501A

to remain in a viable conformation for ligand binding. Once the AhR is bound by a ligand molecule, it translocates into the nucleus and dissociates from the chaperone proteins. The AhR then forms a dimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). This dimer then binds to specific DNA sequences termed dioxin-response elements (DRE) which are located in the 5'-regulatory region of DRE responsive genes (Denison and Nagy, 2003). The AhR battery of genes currently includes cytochrome P4501A dependent monooxygenases (CYP1A), CYP1B1, NADPH: oxidoreductase, aldehyde dehydrogenase, UDP glucuronosyltransferase, and glutathione s-transferase (Nebert et al., 2000), but the list continues to increase as more data becomes available. There are many other genes currently under investigation for regulation by AhR, including CYP1C1 and CYP1C2, which appear to be exclusive to fish species (Godard et al., 2005; Wang et al., 2006; Jönsson et al., 2007a, b), as well as anti-angiogenic factors such as Angz, cardiac myosin isoforms, transforming growth factor β (TGF-β), and genes involved in cardiac cell cycle progression in fish (Bello et al., 2004; Handley-Goldstone et al., 2005; Carney et al., 2006). CYP1A and CYP1B1 expression has been shown to increase after AhR agonist exposure in the murine heart (Nebert et al., 2000) and in the zebrafish heart (Carney et al., 2006). Furthermore, CYP1A has been shown to be significantly active in both mammalian and fish vascular endothelial cells (Van Veld et al., 1997; Kerzee and Ramos, 2001; Dong et al., 2002; Teraoka et al., 2002) and, to a much lesser extent, in vascular smooth muscle (Kerzee and Ramos, 2001) after AhR agonist exposure. In contrast, CYP1B1 has significant expression in vascular smooth muscle but little in the endothelium (Kerzee and Ramos, 2001) after AhR agonist exposure.

There is only one subtype of the AhR in mammals however in most fish species, there are at least two separate genes encoding AhR (Andreasen et al., 2002a, b; Karchner et al., 2005; Antkiewicz et al., 2006). In zebrafish, three AhR genes, designated AHR1a, AHR1b, and AHR2, have been characterized (Karchner et al., 2005). Sequence comparisons indicate that AHR1a in fish has significant sequence homology to mammalian AHR (Andreasen et al., 2002a). Functionally, AHR2 (Andreasen et al., 2002a, b; Karchner et al., 2005; Antkiewicz et al., 2006) and AHR1b (Karchner et al., 2005) exhibit high-affinity TCDD binding, whereas AHR1a appears to lack the ability to bind or be activated

by TCDD or related compounds (Andreasen et al., 2002a; Karchner et al., 2005). The AHR1b gene binds to TCDD with lower affinity than does AHR2, but both may be involved in cell-specific CYP1A regulation in fish (Karchner et al., 2005). Along with the three AHR genes, zebrafish also have two ARNT genes (ARNT1 and ARNT2) (Karchner et al., 2005; Antkiewicz et al., 2006). Antkiewicz et al. (2006) showed that most, if not all toxic responses to TCDD exposure in zebrafish appear to be mediated by AHR2 and ARNT1. Knocking down AHR2 or ARNT1 expression in zebrafish embryos appears to protect them from classic TCDD-induced developmental deformities including pericardial edema, lower jaw malformation, altered heart morphology, decreased stroke volume, and decreased cardiac output (Cantrell et al., 1998; Karchner et al., 2005; Antkiewicz et al., 2006; Billiard et al., 2006).

1.3 Benzo(a)pyrene

Benzo(a)pyrene (BaP) belongs to a family of compounds called polycyclic aromatic hydrocarbons (PAHs). The PAHs are products of incomplete combustion and are found in petroleum products, coal tar, tobacco smoke, vehicle exhaust fumes, and charbroiled foods (Hattemer-Frey and Travis, 1991). There has been an increase in PAH concentrations in aquatic environments around urban areas recently, thought to be associated with urban sprawl and global transport (Van Metre et al., 2000; Borghesi et al., 2008). There is also evidence to indicate that PAHs from oil spills can remain in sediments at detectable levels for decades after the spill site has been decontaminated (Reddy et al., 2002). Fish are exposed to BaP and other related compounds primarily through dietary means, gill uptake, or absorption in incubating eggs from contaminated sediments. BaP is rapidly metabolized and produces toxic effects by activating the AhR, increasing transcription of the CYP1A and CYP1B1 genes (Shertzer et al., 2004). Benzo(a)pyrene stimulates its own clearance from an organism by inducing CYP enzymes that metabolize it. The metabolites produced by CYP activity on BaP are unstable (i.e. epoxides and quinones), and produce mutagenic and cytotoxic effects, as well as oxygen free radicals (Miller and Ramos, 2001). Fish are

most susceptible during the developmental stages of their lifecycle and exposure to BaP during these stages leads to numerous physical deformities including dorsal curvature, difficulties with or failure of swim bladder inflation, and embryonic pericardial and yolk-sac edema (Incardona et al., 2004). However, the role of AhR stimulation compared to other mechanisms of PAH-mediated developmental defects remains unclear (Incardona et al., 2004).

1.4 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) belongs to the halogenated aromatic hydrocarbon (HAH) family of compounds. TCDD has the highest potency and binding affinity for AhR of all the chemicals in this class (Mimura and Fujii-Kuriyama, 2003). Unlike PAHs, it is proposed that all adverse biological responses seen upon exposure to HAHs such as TCDD are caused by AhR activation and CYP induction (Cantrell et al., 1998; Bello et al., 2004; Antkiewicz et al., 2005). Induction of CYP enzymes by TCDD has been shown to increase vascular permeability and vascular lesions (Cantrell et al., 1998; Guiney et al., 2000; Dong et al., 2002). Unlike BaP, the metabolically stable TCDD does not induce its own metabolism although TCDD does bind to CYP substrate binding sites thereby preventing CYP action on other substrates and uncoupling the CYP-associated electron transport chain to produce oxygen free radicals (Schlezinger et al., 1999, 2000b). Fish are highly sensitive to HAH toxicity, especially in the larval and developmental stages. Vascular lesions that develop in the embryonic stage increase in severity during development, eventually resulting in decreased blood flow, vascular bed failure, and tissue necrosis (Henry et al., 1997; Cantrell et al., 1998; Andreasen et al., 2002b; Bello et al., 2004; Antkiewicz et al., 2005). Zebrafish (used as a model species in this thesis) are a less sensitive species than lake trout or other salmonids but still develop characteristic symptoms of AhR-mediated toxicity (Antkiewicz et al., 2005; Goldstone and Stegeman, 2006).

1.5 Cardiac Function and Development

Mammalian and fish hearts have similar forms. Blood flows into the atrium from a major vein and then moves into the ventricle where it is delivered to the aorta (Thisse and Zon, 2002). Cardiac valves form at atrial and ventricular boundaries to direct blood flow and ensure unidirectionality (Dooley and Zon, 2000; Thisse and Zon, 2002). Compared to the four-chambered mammalian heart, the more primitive fish heart has only 2 chambers (Figure 1.2) (Thisse and Zon, 2002). As blood exits the ventricle via the dorsal aorta, it is pumped over the gill arches where oxygenation occurs before the blood is distributed to the rest of the body (Hu et al., 2000).

Heart rate tends to vary with animal size, thus, smaller animals, such as zebrafish, have naturally fast heart rates (Hu et al., 2000, 2001). There are several natural stressors that affect heart rate, such as temperature and oxygen fluctuations or predation. Increased stress leads to the release of endogenous catecholamines epinephrine and norepinephrine which are known to increase heart rate in both mammals and fish (Eckstein et al., 1962; Cobb and Santer, 1973; Denvir et al., 2008). Likewise, the endogenous neurotransmitter acetylcholine decreases heart rate in both mammals and fish (Cobb and Santer, 1973; Yang et al., 1993).

Regulation of cardiac function in adult fish lags behind in understanding compared to mammals. Furthermore, the point at which developing hearts become responsive to catecholamines and acetylcholine is unclear. There has been a consistent toxicological effect noted after developmental TCDD exposure in fish, namely decreased heart rate and cardiac output (Andreasen et al., 2002b; Antkiewicz et al., 2005; Carney et al., 2006; Yamauchi et al., 2006). However, whether this is due to a change in endogenous cardiac regulation, delayed development, or a structural change is unclear.

Normal cardiovascular development in zebrafish begins with the formation of a primitive heart tube composed of cardiac myocytes and endocardial endothelial cells (Figure 1.3; Nemer and Nemer, 2001). This heart tube begins beating around 22 hours post-fertilization (hpf), with coordinated atrial and ventricular contractions coinciding with heart looping that occurs around 36 hpf (Dooley and Zon, 2000; Goldstone and Stegeman,

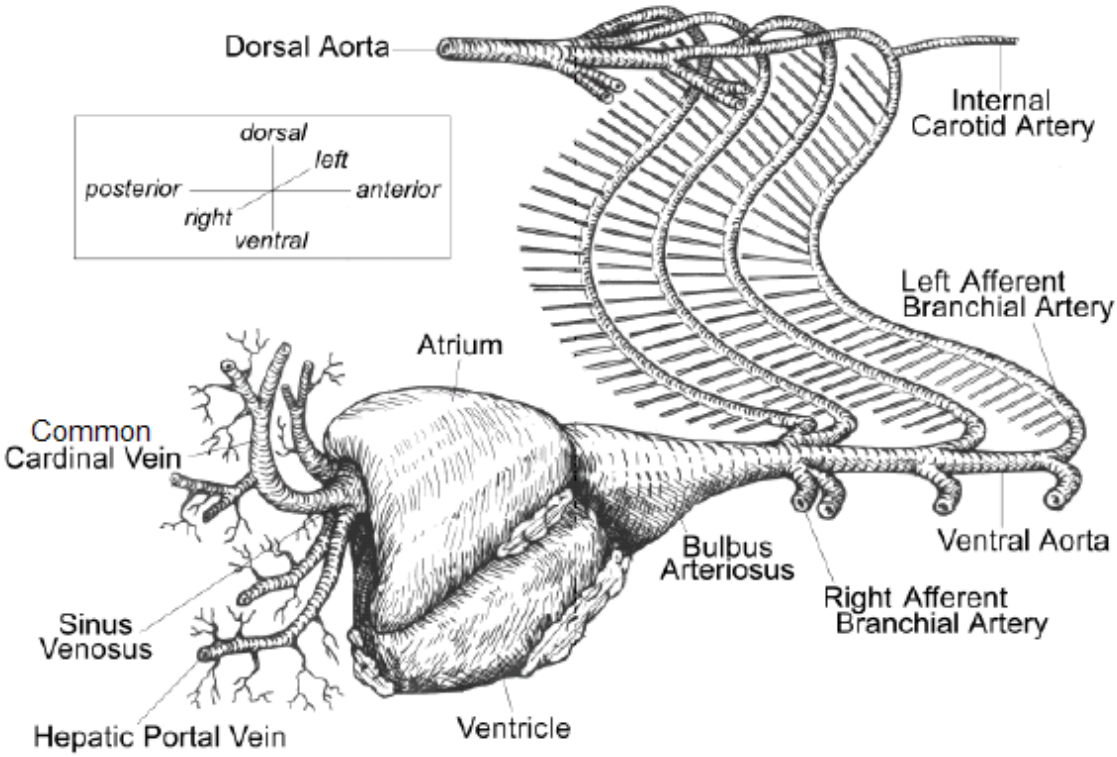


Figure 1.2: Diagram of the adult zebrafish heart (Hu et al., 2001; reproduced with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.).

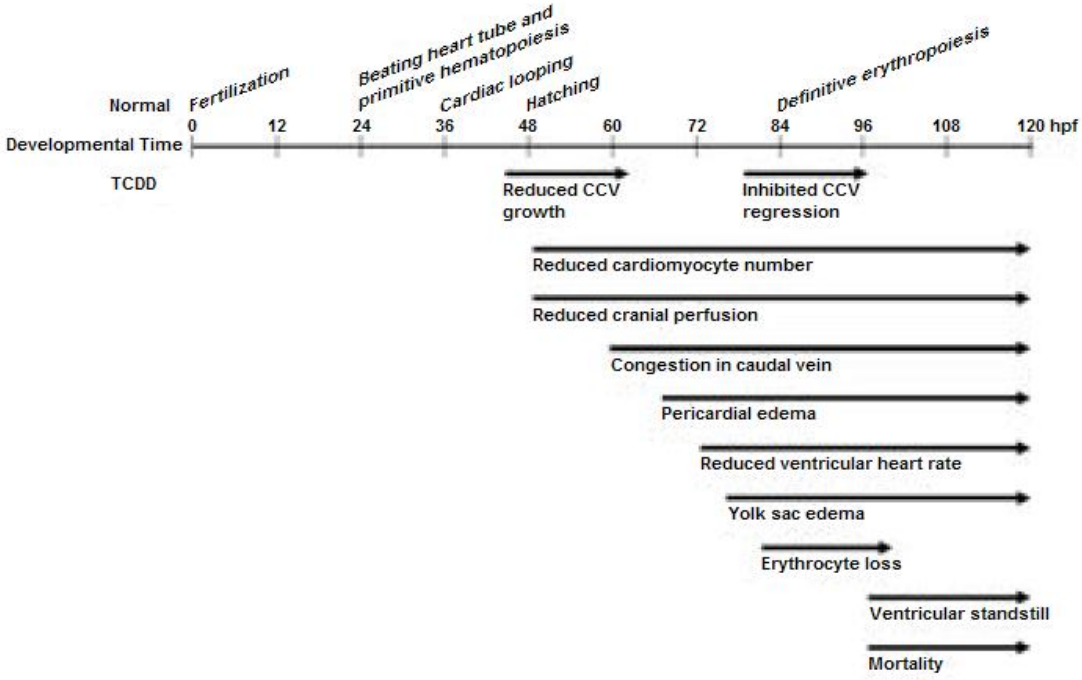


Figure 1.3: Time-line of normal zebrafish development from 0 to 120 hours post-fertilization (hpf) and effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on normal development. CCV – common cardinal vein (Goldstone and Stegeman, 2006; reproduced with permission from Taylor & Francis).

2006). Hatching occurs over the course of the third day after fertilization and definitive erythropoiesis occurs before the fourth day. It is during these first 96 hours of development that exposure to chemicals such as TCDD can induce circulatory impairment (Belair et al., 2001). Signs of circulatory impairment begin to appear around 48 hpf with the reduction of common cardinal vein growth and subtle reductions in red blood cell perfusion rate in the brain and trunk (Dong et al., 2002; Teraoka et al., 2002; Bello et al., 2004). While these initial indicators appear to involve only vasculature, there is a characteristic cardiac phenotype associated with circulatory impairment. Before alterations in blood flow are apparent, the looping process of the heart is affected. Normal looping occurs at 36 hpf, but developmental exposure to polycyclic and halogenated aromatic hydrocarbons result in poorly looped or unlooped hearts (Figure 1.4) which may be a major contributing factor to the eventual circulation failure that precedes mortality after exposure to these chemicals (Antkiewicz et al., 2006; Carney et al., 2006; Incardona et al., 2006).

1.6 Vascular Function and Development

Arterial physiology shows many similarities in all vertebrates. All artery walls are composed of three layers: a single layer of endothelial cells lining the vessel lumen, a layer of smooth muscle, and a layer of connective tissue. The endothelial layer is involved in many physiological activities, including vasoconstriction and dilation, thrombosis, atherosclerosis, angiogenesis, inflammation, nitric oxide functions, as well as controlling passage of material into and out of the bloodstream. The smooth muscle varies in thickness depending on the size and flow through the vessel and is responsible for vasoconstriction and vasodilation. Nerves and lymphatic vessels are embedded in the outermost layer which also provides support for the blood vessels while attaching them to the surrounding tissue (Seeley et al., 2003).

Nitric oxide (NO), a small free radical molecule, is produced by a two step reaction involving the breakdown of L-arginine which is catalyzed by the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS: a neuronal form (nNOS), an inducible

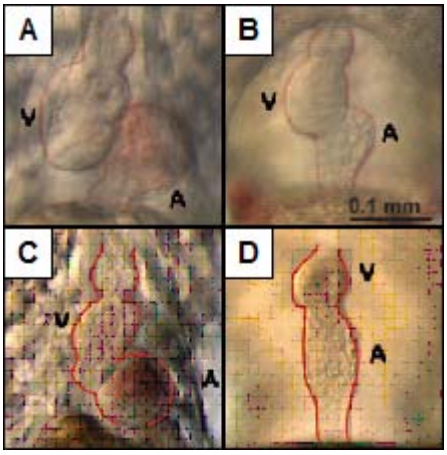


Figure 1.4: Developing zebrafish hearts at 48 (A & B) and 96 (C & D) hours post-fertilization exhibiting either typical looping after exposure to dimethyl sulfoxide (DMSO) (A & C) or lack of looping (B & D) due to exposure to 1 ng/ml (B) or 0.4 ng/ml (D) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin for 1 hour after fertilization. V – ventricle; A – atrium (adapted from Antkiewicz et al., 2006 and Carney et al., 2006; reproduced with permission from Oxford University Press and the American Society for Pharmacology and Experimental Therapeutics).

form (iNOS), and an endothelial form (eNOS), the latter of which is responsible for much of the nitric oxide production in endothelial cells of the mammalian vasculature (Vallance and Chan, 2001; Swenson et al., 2005). Nitric oxide has a wide array of effects on the mammalian vasculature, including vasodilatory, anti-thrombotic, anti-inflammatory, and anti-proliferative effects, as well as inhibitory effects on vasocontrictive agents such as angiotensin II (Vallance and Chan, 2001).

Prostanoids are also involved with many physiological functions including vasoconstriction and vasodilation (Breyer et al., 2001; Bos et al., 2004; Alfranca et al., 2006). There are five basic naturally occurring bioactive prostanoids, all of which are metabolites of arachidonic acid. The five primary prostanoids are prostaglandins D-2 (PGD_2), E-2 (PGE_2), and F-2 alpha ($PGF_{2\alpha}$), prostacyclin (PGI_2), and thromboxane (TxA_2) (Figure 1.5). Each prostanoid has a specific G-protein-coupled receptor to which it binds with high affinity, although some cross reactivity is observed (Breyer et al., 2001; Bos et al., 2004; Alfranca et al., 2006). $PGF_{2\alpha}$ and thromboxane have been shown to cause constriction of blood vessels by activating pathways that increase smooth muscle intracellular calcium concentrations. In contrast, PGD_2 and PGI_2 cause relaxation of blood vessels by stimulating pathways that result in increased cyclic adenosine monophosphate (cAMP) levels. There are four receptor subtypes for PGE_2 ($EP_{1\text{-}4}$) that, when ligand bound, produce different effects. When the EP_1 receptor is activated, IP_3 is generated, cell calcium concentrations increase, and vasoconstriction ensues. EP_2 and EP_4 receptors signal pathways that activate the cAMP cascade in smooth muscle and cause vasodilation. The EP_3 receptor uses a pertussis toxin-sensitive G-protein-coupled mechanism to inhibit cAMP production, leading to vasoconstriction (Breyer et al., 2001; Bos et al., 2004; Alfranca et al., 2006). Pharmacological agents such as indomethacin, naproxen, phenylbutazone, salicylates, and non-steroidal anti-inflammatory drugs (NSAIDs), all inhibit prostanoid synthesis (Curtis-Prior, 1976). The effects of NSAIDs on vascular contractility will vary, but would depend on which prostanoid was being produced and which receptors were present on a particular vascular smooth muscle tissue.

Although they are products of different metabolic pathways, nitric oxide and prostaglandins have similar functions (Vassalle et al., 2003). It is likely that there is some

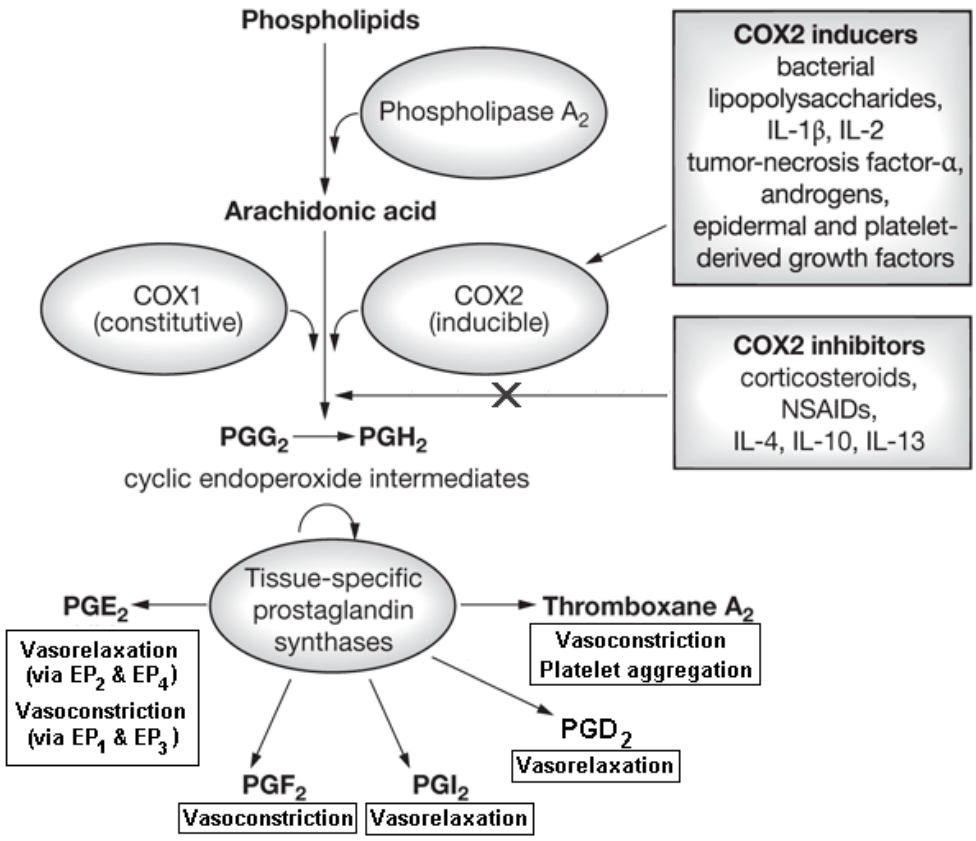


Figure 1.5: Prostaglandin synthesis, platelet inhibition, and functions (adapted from Zha et al., 2004; reproduced with permission from Elsevier).

interaction between the two pathways and there is evidence that manipulation of one will affect the other (Vassalle et al., 2003). Vassalle et al. (2003) demonstrated that PGE_2 and 6-keto-$PGF_{1\alpha}$ (a stable metabolite of $PGF_{2\alpha}$) levels in human microvascular endothelial cells significantly increased when NO concentrations were increased. This study also found that NO release was enhanced when cells were treated with indomethacin, but that PG levels did not increase after pharmacological inhibition of NOS. It was suggested that indomethacin treatment increased intracellular calcium levels and that PGs helped regulate NOS activity by controlling the availability of calcium (Vassalle et al., 2003). Conversely, a similar study in porcine aortic endothelial cells found that increased NO concentrations significantly decreased PGI_2 production (Takeuchi et al., 2004). This study also found that high NO concentrations inhibited calcium entry into the endothelium, which suggests that excessive NO limits PGI_2 production by modifying the availability of calcium to phospholipase A_2. The exact influences that NO has on PG pathways, and vice versa, are still uncertain, but it is very clear that there is some interaction between them.

1.7 Normal Mammalian Vasorelaxation

Normal mammalian vasorelaxation is regulated predominantly by nitric oxide. Endothelial cells lining blood vessels continuously produce nitric oxide to maintain and control vascular tone (Ignarro, 1990; Moncada et al., 1991), however prostaglandins may become the dominant influence on vasoactivity during inflammatory reactions. Although prostaglandins are also being constitutively produced, their influence is less than nitric oxide. Prostaglandins exist in a specific ratio but when synthesis is stimulated by hormones or inflammation, the ratio changes. For example, PGI_2, a potent vasodilator and platelet aggregation inhibitor, and TxA_2, a vasoconstrictor and platelet aggregation stimulator, are constantly produced in a balance to help maintain blood flow and prevent clotting. If the ratio of PGI_2/TxA_2 is high, vasodilation and anti-platelet activity are promoted. Therefore, although prostaglandins are involved in many complex reactions, it is nitric oxide that is

responsible for most control of vascular tone in mammals (Ignarro, 1990; Moncada et al., 1991).

1.8 Arterial Relaxation in Fish

Currently there is controversy surrounding the issue of arterial relaxation in fish. There is extensive information available on the structure and function of mammalian blood vessels but little is known regarding fish vasculature. Studies on fish vasculature have shown that familiar mammalian vasoactive factors have opposite effects in fish. Most notably, whereas acetylcholine dilates blood vessels in mammals, it contracts blood vessels in trout and dogfish (Farrell and Johansen, 1995). Furthermore, although the specific regulators of vascular tone have not been conclusively identified, evidence indicates that cyclooxygenase-derived compounds primarily control vasoconstriction and dilation (Farrell and Johansen, 1995; Park et al., 2000).

Digital motion analysis has been used to monitor dorsal arteries and veins of zebrafish larvae and document responses to vasoactive compounds (Fritsche et al., 2000). This study found that the NO-donor sodium nitroprusside (SNP) induced vasodilation and the NOS-inhibitor, N-Nitro-L-Arginine Methyl Ester (L-NAME), induced vasoconstriction suggesting that the vasculature was influenced by endogenous NO. Whole mount immunoreactivity assays for eNOS and nNOS identified the presence of eNOS in dorsal vein endothelial cells and the absence of nNOS in the peripheral tissues. However, this must be interpreted with caution. The investigators used polyclonal antibodies derived from the human and bovine eNOS genes and the rat nNOS gene (Fritsche et al., 2000). Cross-reactivity of fish tissue with polyclonal antibodies to eNOS does not definitively prove eNOS is present in fish and it is possible that the antibodies simply bound to similar, unspecified proteins. In fact, a recent review has suggested that it is actually nNOS that is the source of NO in fish vasculature (Toda and Ayajiki., 2006). Therefore, although evidence of a functional role of NOS in fish vasculature has been detected, evidence of eNOS is lacking.

Tissue bath experiments have been used to expose piscine vessels to known mammalian endothelial-mediated vasorelaxants, several nitric oxide or NOS antagonists, various prostaglandins with COX and NOS inhibitors, and prostaglandin precursors with antagonists (Farrell and Johansen, 1995). This study found that adenosine, adenosine diphosphate, adenosine triphosphate, and acetylcholine, which stimulate vasodilation in mammals, cause constriction of isolated fish vessels. Thrombin and bradykinin had no effect and the prostaglandin precursors elicited a limited response. Both PGE_2 and PGI_2 produced strong relaxations in fish vessels and were found to completely antagonize precontraction from $PGF_{2\alpha}$. Investigators could not identify a non-prostanoid compound that could be responsible for endothelium-mediated responses, suggesting that there were no biologically significant nitric oxide-mediated coronary artery responses in the species tested (Farrell and Johansen, 1995).

A second, similar study investigated the different responses in aorta from carp and rats exposed to vasoactive compounds (Park et al., 2000). A donor-detector system in which a section of rat aorta was used as a relaxing factor donor and a section of carp aorta was hung in the same bath to detect responses to any substance released by the rat aorta. Individual vessels from the carp failed to react to both direct NO donors and indirect NO stimulating agents while all of these substances produced obvious vasorelaxation in rat aorta. Increasing intracellular calcium using the calcium ionophore A23187 relaxed carp aorta, even in the presence of NOS inhibitor, L-NAME while indomethacin exposure and endothelium denudation inhibited the effects of A23187 (Park et al., 2000). When the donor-detector system was reversed, it failed to find evidence of NO production in carp aorta. From these results, the investigators suggested that a cyclooxygenase metabolite is responsible for endothelium-derived vasorelaxation in carp. However, they could not completely rule out the possibility that the vessels produced NO or that these results are indicative of responses in all fish (Park et al., 2000).

The results of these studies do not provide substantial evidence to support nitric oxide relaxation in fish, despite the immunoreactivity observed in developing zebrafish (Fritsche et al., 2000). There appears to be stronger evidence in support of a more biologically significant role for prostanoid-mediated relaxation in fish arteries (Farrell and

Johansen, 1995; Park et al., 2000). Further studies are needed to clarify whether species differences, developmental differences, or artery bed differences have prevented a clear consensus on the relative roles of NO versus prostanoids in fish vasorelaxation.

1.9 Zebrafish as a Model for AhR-Mediated Toxicity

Zebrafish are an efficient and useful model for toxicity studies. Being small in size, it is easy to maintain large numbers of test animals with a small amount of space. They produce high numbers of eggs and the embryos develop rapidly. There is vast knowledge on the normal development of zebrafish and it is therefore possible to study what causes deviations from normal. Relevant to this study, the progression of cardiovascular development and how TCDD affects this process has been well documented (see Goldstone and Stegeman, 2006 for review) (Figure 1.3). The vasculature and circulation of the developing zebrafish has also been very well mapped out (Lawson and Weinstein, 2002). While the fish are in the early stages of development, they have transparent bodies which allow for the use of standard microscopy procedures to view the heart as it beats. The Wellcome Trust Sanger Institute, in collaboration with three other laboratories, have completely sequenced and annotated the zebrafish genome providing a wealth of genetic and biological information. Finally, zebrafish are reasonably sensitive to the AhR agonists TCDD and BaP, and have been used to characterize mechanisms of AhR-mediated developmental toxicity (Van Veld et al., 1997; Andreasen et al., 2002b; Bello et al., 2004; Antkiewicz et al., 2005), making them ideal models for this study.

1.10 AhR and Cardiovascular Toxicity

Aryl hydrocarbon receptor-mediated toxicity in developing fish is characterized by yolk sac edema, hemorrhaging, severe cardiovascular deformities, impaired swim bladder

inflation, and craniofacial malformations (Cantrell et al., 1998; Andreasen et al., 2002b; Brinkworth et al., 2003; Karchner et al., 2005; Antkiewicz et al., 2006; Billiard et al., 2006). Lethality is usually due to cardiovascular defects and eventual complete circulation failure (Figure 1.3; Henry et al., 1997). In normal vascular bed formation, the endothelial cells migrate and form the framework for the vasculature. Once in place, the endothelium secretes chemical signals that initialize smooth muscle and connective tissue growth (Drake, 2003; Ferguson et al., 2005). If the endothelial cells are damaged or unable to function properly, vascular bed development would be impaired. It is proposed that increased CYP expression and activity increase expression of the anti-oxidant response element transcription factor called nuclear erythroid 2 p45-related factor 2 (NRF2), reactive oxygen species, and lipid membrane damage to produce symptoms of developmental AhR-mediated toxicity (Andreasen et al., 2002a; Timme-Laragy et al., 2009). However, the precise mechanism by which these events are linked to produce developmental AhR-mediated cardiovascular deformities is not clear.

Currently, opinions are divided in the scientific community regarding whether the vasculature or the heart is the primary target of AhR-mediated cardiovascular toxicity. There are many studies which support the idea that the heart is the primary target for damage and the other symptoms, such as reduced blood flow and edema, are secondary to cardiac failure (Incardona et al., 2004; Antkiewicz et al., 2005; Carney et al., 2006). For example, a poorly looped or unlooped heart is common after TCDD exposure (Teraoka et al., 2002; Antkiewicz et al., 2006; Carney et al., 2006; Incardona et al., 2006). It is believed that this cardiac phenotype leads to decreased cardiac output resulting in decreased blood flow (Henry et al., 1997; Belair et al., 2001; Dong et al., 2002, 2004; Teraoka et al., 2002). There is also evidence to support the idea that cardiac dysfunction is a secondary effect. Dioxin was observed to induce local circulation failure and reduce blood flow in the brain which preceded most other symptoms of TCDD toxicity including cardiac symptoms (Dong et al., 2002). Another study reported that blood flow in the head and gills of developing zebrafish larvae was reduced before a reduction in heart rate (Andreasen et al., 2002b). Since vascular permeability increases during development after TCDD exposure (Cantrell et al., 1998; Guiney et al., 2000; Andreasen et al., 2002b), it is possible that the

vascular endothelium is a primary target or at least an independent target from the heart for TCDD-induced toxicity. Damage to the endothelium could cause unregulated vasoconstriction or vasorelaxation, as well as potentially altering vascular bed development, which may explain some of the TCDD-induced cardiovascular toxicity.

1.11 mRNA Expression as an Indicator of AhR Ligand Exposure

Induction of cytochrome P450 enzyme mRNA expression, particularly CYP1A, has traditionally been used as a biomarker for quantifying AhR agonist exposure but CYP1A may also be an indicator of effect. Induction of the detoxification enzymes may not be the only cause of pathological effects seen with exposure (Handley-Goldstone et al., 2005; Carney et al., 2006; Teraoka et al., 2009). Although mRNA may be increased, enzyme activity may not change (Wassenberg and Di Giulio, 2004; Timme-Laragy et al., 2007). However, there may be other genes whose expressions are increased which, in turn, produce pathological effects on the vasculature such as COX and iNOS. Understanding the time-course of individual isozymes changed and the identity of all relevant genes that are altered after AhR agonist exposure is important in determining a more concise mechanism of AhR agonist toxic effects. There is abundant evidence supporting a role for the AhR pathway in HAH and PAH toxicity, but the mechanism of toxicity remains unclear (Goldstone and Stegeman, 2006). Comparing mRNA expression to the degree of varying developmental abnormalities may indicate phenotypes associated with specific AhR-mediated alterations in mRNA. Most mechanistic studies to date have used mortality or gross physical malformations elicited by very high levels of AhR agonists as endpoints for toxicity. However, in order to dissect whether cardiac or vascular tissues are the primary target, and whether CYP1A is required, lower AhR agonist concentrations need to be used. Furthermore, it is unclear at what point the developmental defects will affect ultimate survival of the fish. For example, evidence suggests even low level developmental PAH exposure can affect adult pink salmon returns for spawning (Heintz et al. 2000). Therefore, mechanistic laboratory studies examining effects of developmental AhR agonists need to be

conducted using lower exposure concentrations as well as examining time points further in development before we can relate these toxicities to ultimate survival. More importantly, due to the large number of genes potentially involved, phenotypic anchoring to these lower concentrations of AhR agonists with similar, overlapping mechanisms is critically needed to determine specific factors involved in AhR developmental toxicity.

1.12 Importance of Understanding Sublethal AhR Agonist Effects

There is ecological concern surrounding the possibility of sensitive fish species being exposed to AhR agonists. There may be unforeseen reproductive consequences and an ultimate decline in fish survival (Heintz et al., 2000, 2007). This poses potential environmental problems involving declining feral fish populations and decreasing game fish availability, but is also an economic problem for fisheries and aquaculture facilities. Very low, environmentally relevant concentrations may severely impact the quality of fish being produced and increase long-term mortality rates. This would lead to increased costs associated with raising and housing fish, as well as a reduction in the product quality being turned out by these types of aquaculture facilities. This thesis aims to provide new evidence to help better determine the mechanism of AhR agonist-induced toxicity which ultimately could be used for setting better regulations for these contaminants.

1.13 Hypotheses

The following were hypothesized for this thesis:

1) Both BaP and TCDD, having similar and overlapping mechanisms of action, will induce similar responses in CYP and COX mRNA expression in zebrafish while the purported antagonists resveratrol and α-naphthoflavone will inhibit any changes induced by these agonists.

2) Changes in CYP and COX mRNA expression will be tissue-specific in adult zebrafish, differing between hepatic and arterial tissues.

3) Changes in CYP and COX mRNA expression will be different between tissues isolated from adult zebrafish and whole larvae homogenates.

4) The AhR-agonist-induced deformed cardiac phenotypes will correlate with alterations in expression of the same genes as vascular abnormalities.

1.14 Research Objectives

The following were the objectives for this thesis:

1) Method and primer development for CYP, COX, and NOS isoforms as well as optimization of *in vivo* and histological larval cardiovascular methods.

2) Determination of effects on tissue-specific mRNA expression in adult zebrafish induced by BaP and TCDD with and without the purported AhR antagonists.

3) Determination of effects on whole larval homogenate mRNA expression in zebrafish at 5 and 10 days post-fertilization after developmental exposure to BaP and TCDD with and without the purported AhR antagonists.

4) *In vivo* and histological analysis of various indicators of cardiovascular health and function in larvae at 10 days post-fertilization after developmental exposure to BaP and TCDD with and without purported AhR antagonists.

5) Perform correlation analysis of *in vivo* and histological markers of cardiovascular phenotype and function with mRNA expression changes after sublethal exposure to AhR ligands.

2.0 Method Development

2.1 Primer Design and Validation

2.1.1 Choosing target genes

The CYP enzymes are commonly used to assess AhR agonist exposure. While many of the CYP isoforms have been used in previous real-time reverse transcriptase polymerase chain reaction (rtrt-PCR) studies, the primers developed for these earlier experiments based on partial gene sequences were not suitable for the purpose of this thesis (Wang et al., 2006; Jönsson et al., 2007a, b; Timme-Laragy et al., 2007). These older primers flanked regions that were approximately 100 basepairs (bp) in length, while the desired length of rtrt-PCR products for these experiments was between 150 and 200 bp. In the case of both CYP1C2 and the COX genes, primers have been published for these genes in zebrafish (Jönsson et al., 2007a; Ishikawa et al., 2007), but were not available at the time these experiments were initiated. Since these experiments were aimed at investigating molecular changes of CYP, COX, and NOS isoforms, primers needed to be designed for use in regular and real-time rt-PCR.

2.1.2 Primer development for CYP and COX isoforms

There were many programs involved in designing the primer pairs used in the real-time polymerase chain reaction (PCR). The process began with searching the National Center for Biotechnology Information nucleotide database for cDNA sequences of the genes of interest. Sequences from two to four organisms other than zebrafish were necessary and tried to include sequences from humans, rats (*Rattus norvegicus*), dogs (*Canis familiaris*), and rainbow trout (*Oncorhynchus mykiss*). These other animals were chosen because they are studied in the Weber laboratory and great care was taken to avoid effects from any possible contamination that may occur. Multiple sequence alignments for the CYP isozymes were acquired through the use of DNAsmac (Toulouse, France), SeqAid

II (Manhattan, KS), and GeneDoc (http://www.psc.edu/biomed/genedoc). The Geneious program (Auckland, New Zealand) was used for the initial sequence alignment for the COX isozymes. After alignments, an online program called the DINAmelt server (http://dinamelt.bioinfo.rpi.edu/quikfold.php) showed the structure of the cDNA sequence under specified conditions. The parameters chosen mimicked the annealing phase of a polymerase chain reaction. These parameters were chosen in order to visualize any folding or hairpin loops that would occur in the sequence at the specific annealing temperature so that primers could be designed in regions where these structural changes did not occur. Once the desired regions were determined, Primer Designer (Cambridge, MA) was used to find at least ten primer pairs that would produce a PCR product around 200 basepairs in size. Each primer was around 20 basepairs in length and had a predicted melting point around 60°C.

The GenBank and primer sequences were transferred into Amplify (Bill Engels, University of Wisconsin) and a virtual PCR was run with each primer pair to determine product size and verify that one fragment was isolated and amplified. Amplify was also used to ensure that the primers designed for one gene did not target part of a sequence from a different gene. Two primer pairs for each gene were then chosen (Table 2.1) and a regular PCR was run using liver and artery samples from control- (corn oil) and BaP-treated (2 g/kg) zebrafish or larval samples from control- (DMSO) and TCDD-treated embryos.

2.1.3 Primer validation

The PCR products were run on a 1% agarose gel to confirm that the actual product obtained was of the predicted size in either adult tissue samples or whole larvae samples (Figure 2.1). Artery and liver tissues were collected from adult zebrafish 24 hr after injection with either 1% v/w corn oil or 2 g/kg BaP. Whole larvae were collected at 10 days post-fertilization (dpf) after being exposed to either 0.5% v/v dimethyl sulfoxide (DMSO) or 10 ng/L TCDD. Candidate primer pairs were chosen from these gels for real-time rt-PCR. In some cases, primer pairs amplified multiple fragments (primer pairs #6

and 11 in Table 2.1) and were no longer used. Real-time rt-PCR analysis was performed in a Stratagene MX3005P QPCR system (VWR, Mississauga, Ontario, Canada) using SYBR Green qPCR Supermix (VWR). The reaction was carried out with the same samples and the chosen primers to validate their effectiveness in rtrt-PCR. The MxPro3005 software provides product melting point plots and a single melting point was used as a further confirmation that one product was produced for each primer pair.

2.1.4 Primer selection for CYP and COX isoforms

Of the primer pairs indicated in Table 2.1, # 2, 3, 5, 7, 9, 10, 13, and 14 were selected and used in all subsequent experiments shown in Table 2.2, as well as in Chapters 3 and 4. Primer efficiencies were calculated in the MxPro3005 software. Briefly, serial dilutions of a single sample were used in real-time rt-PCR and a plot of sample dilution versus threshold value, that is, the cycle point at which amplification reaches a logarithmic phase, was plotted on a logarithmic scale. The slope of the plot for each gene was used to calculate the efficiencies using the equation:

$$\text{Efficiency (\%)} = (10^{(-1/\text{slope})} - 1) * 100$$ (Table 2.2).

Generally, if the efficiencies of the primer pairs for the housekeeping and target genes differ by more than 5%, a two-fold difference could be observed as an artifact. However, since all the efficiencies calculated were greater than 100% they are all binding at maximum capacity. Therefore, the differences observed in primer pair efficiencies should be negligible. Based on a single melting peak in rtrt-PCR, the presence of a single band on agarose gels and suitable rtrt-PCR efficiencies, the chosen primer pairs for CYP, COX, and G3PDH were considered to be suitable and PCR conditions considered to be optimized (Table 2.2).

2.1.5 Primer development for nitric oxide synthase isoforms

One of the original goals of this set of studies was to analyze nitric oxide synthase (NOS) expression, specifically, endothelial NOS (eNOS). Personal correspondences with the Wellcome Trust Sanger Institute, the organization responsible for sequencing the

Table 2.1: Primers designed for each cytochrome P450 (CYP) and cyclooxygenase (COX) gene of interest and the expected fragment size. G3PDH was developed as a housekeeping gene. bp – basepairs

Pair #	Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Fragment Size
1	CYP1A	698s - TTCGGGAAGATCGTGGGCAG	932as - TTCGGTCTTCGCAGTGGTTGA	208 bp
2	CYP1A	745s - TGCCGATTTCATCCCTTTCC	932as - TTCGGTCTTCGCAGTGGTTGA	179 bp
3	CYP1B1	1499s - AGACCTGACCACCAACGTGC	1718as - ATGTTCCTACCAAGCTGTCC	239 bp
4	CYP1B1	1543s - GATGCATTGGAGAAGACG	1727as - GAGTCTGAGATGTTCCTACC	204 bp
5	CYP1C1	1542s - CAGATTCCTGGATGAGAACG	1765as - CCTCTGAGCTTGGCGGAGAT	243 bp
6	CYP1C1	1613s - AGCGGAGGTGCATTGGTGAG	1794as - ATGCCGGTGAAACCAAGCCA	201 bp
7	CYP1C2	161s - GATAGCATGGCGCAGTCGGA	322as - TTCCAACAAGCGGCCAAGCA	181 bp
8	COX-1	259s - GGGATCTGTGTTCGATATGG	440as - CCAATCCCTGAGGAACGACC	201 bp
9	COX-1	829s - GGAGATTCTCTTGATCGCCA	1031as - CTCACGAAGCCACAAGGTAG	222 bp
10	COX-2a	777s - CAGGTTGTGGATGGTGAGGT	1029as - CACCAATCAGGATGAGACGA	253 bp
11	COX-2a	1341s - GCAGTTCAAGGAGTCGCTGT	1522as - CACAAGAAGGCCAGGATACA	201 bp
12	COX-2b	567s - CCAGATCCTCAACGCACCAA	764as - CCTCACCATCCAGAATCTGA	217 bp
13	COX-2b	1076s - GCTCAAGTTTGATCCCGAAC	1265as - GCTGTTGACGCCATAATCTGT	190 bp
14	G3PDH	586s - AGCACTGTTCATGCCATCAC	701as - TACTTTGCCTACAGCCTTGG	135 bp

Table 2.2: Primer efficiencies for primer pairs chosen for each gene of interest.

Gene	Primer Pair	Slope	Efficiency (%)
CYP1A	2	-3.153	107.6
CYP1B1	3	-3.321	100.0
CYP1C1	5	-3.270	102.2
CYP1C2	7	-3.148	107.8
COX-1	9	-3.086	110.9
COX-2a	10	-2.807	127.1
COX-2b	13	-3.148	107.8
G3PDH	14	-3.109	109.7

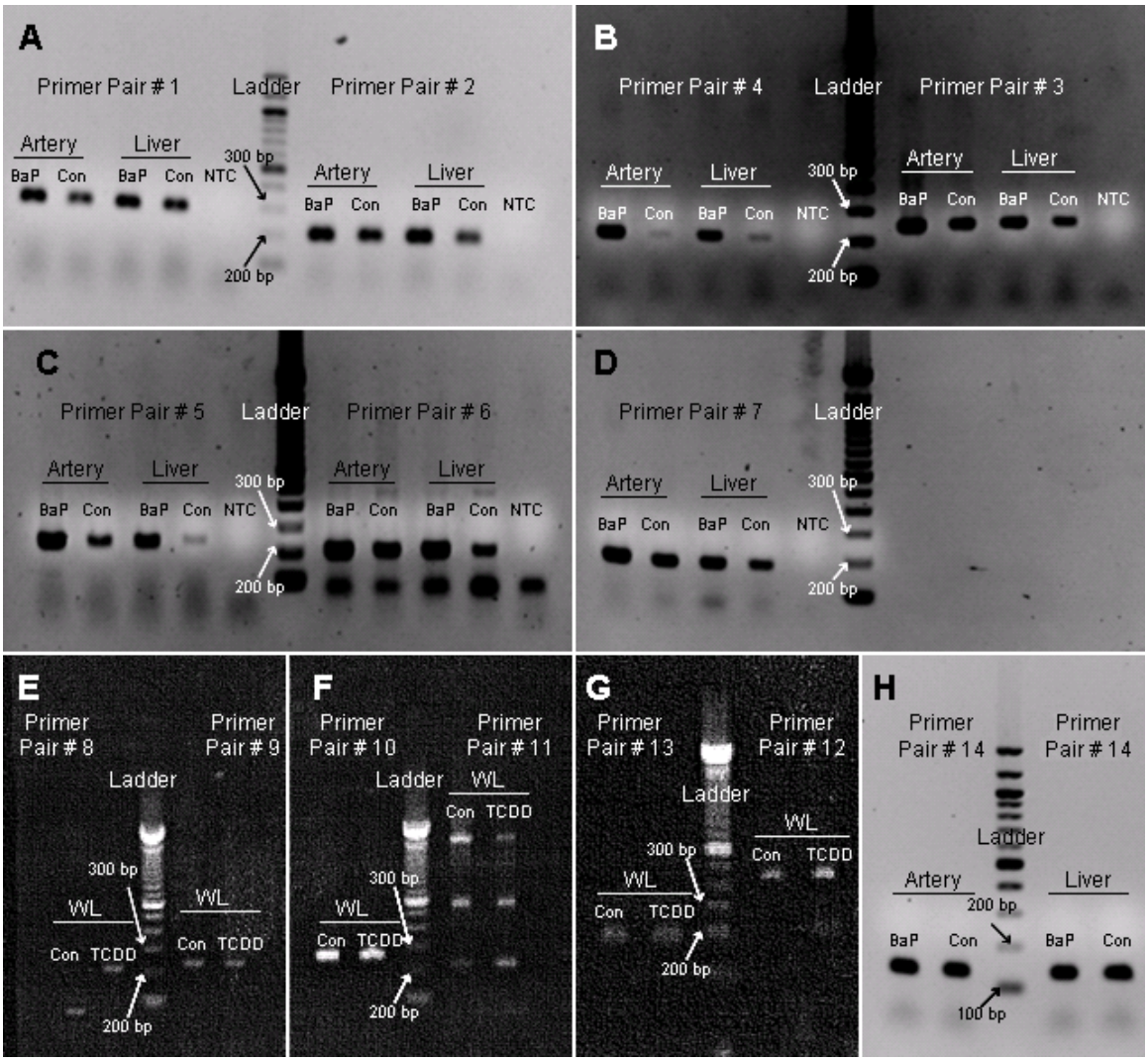


Figure 2.1: Agarose gel images of PCR fragments from primer validation for (A) cytochrome P450 (CYP) 1A, (B) CYP1B1, (C) CYP1C1, (D) CYP1C2, (E) cyclooxygenase (COX) 1, (F) COX-2a, (G) COX-2b, and (H) glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Artery and liver tissues were collected from adult zebrafish 24 hr after injection with either corn oil or 2 g/kg BaP. Whole larvae were collected at 10 days post-fertilization (dpf) after being exposed for 96 hours to either 0.5% v/v dimethyl sulfoxide (DMSO) or 10 ng/L TCDD. DNA ladder gradations are labeled in basepairs (bp). BaP – benzo(a)pyrene; Con – control; TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WL – whole larvae; NTC – no template control.

zebrafish genome, revealed that zebrafish do not have the eNOS gene. In support of this, a previous study failed to find evidence of the presence of a gene encoding for an eNOS homologue in the giant shovelnose ray (*Rhinobatus typus*) (Donald et al., 2004). The two other isoforms of NOS that are known to be present in the zebrafish genome are inducible NOS (iNOS) and neuronal NOS (nNOS). Since beginning this project, nNOS in fish has been postulated to fulfill much of the role played by eNOS in mammals (Toda and Ayajiki, 2006). However, iNOS was pursued in addition to nNOS because of its known role in inflammatory responses (Swenson et al., 2005) which may be relevant in AhR-mediated toxicity. Primers were developed following the previously described methods and validation was attempted (Table 2.3).

2.1.6 iNOS and nNOS primer validation

During the initial primer development, there was only a partial cDNA sequence available for the iNOS gene. The first primer pair (# 15) produced multiple products in real-time rt-PCR but the second pair (#16) appeared to amplify only one fragment based on a single melting peak. However, the PCR product of the predicted size was only being produced in BaP-exposed arterial tissue while a second unknown fragment was also amplified in all the samples (Figure 2.2). Three new primer pairs (#17-19) were later designed for iNOS after this and properly validated. With these latter three primer pairs, each amplified a single product this time, but now all three fragments were larger than the predicted values (Figure 2.2). Fragments from the first two iNOS primer pairs were sent out to be sequenced but sequencing was unsuccessful. Of the two primer pairs designed for nNOS, one pair had multiple products and the other pair amplified fragments that were much smaller than the predicted fragments (Figure 2.3). At this point, due to time constraints and a shifting focus of this thesis to CYP and COX gene expression, resolution of the NOS primer problems was not further pursued, leaving this project without any successful NOS primers. Furthermore, although the zebrafish genome is completely sequenced, the annotation is lagging behind. Better success with designing NOS primers will be aided once these genes are annotated.

Table 2.3: Primers designed to amplify inducible and neuronal nitric oxide synthase (NOS). bp – basepairs

Pair #	Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Fragment Size
15	iNOS	55s - ACCAGACTGATCCATGGCTA	193as - GCATAGAGAACAGTACAGCG	158 bp
16	iNOS	108s - GAGATGCAAGGTCAGCTTCA	249as - GGTTGAGCATGGTGTTCAGT	161 bp
17	iNOS	832s - TGATGTGCTTCCACTAGTCC	1035as - TGTGCCCATGTACCAGCCGT	223 bp
18	iNOS	1347s - CCGTCTATCATCAGGAGTTG	1570as - TGGCAAAGCTCAGTGACTTC	243 bp
19	iNOS	2434s - TCCACCTTCACAAAGCCTGC	2608as - TCTGACTCAGGATGAAGGCG	194 bp
20	nNOS	801s - CAACATGCCCACGGTGCTTA	997as - CCTATGCAAACGTGCTCAGT	216 bp
21	nNOS	1040s - ACGCTCACGAACCAGATGAA	1255as - ATTCGTCCCACACAGCGAGC	235 bp

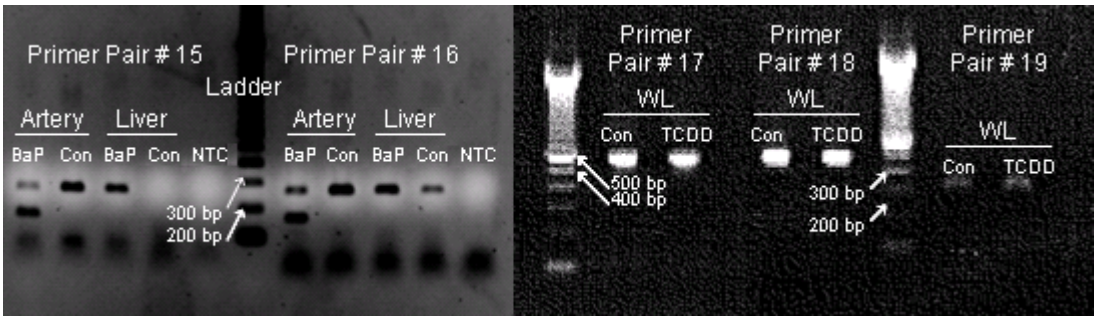


Figure 2.2: Agarose gel to validate primer pairs (pairs #15 – 19 from Table 2.3) for inducible nitric oxide synthase (iNOS). DNA ladder gradations are labeled in basepairs (bp). Artery and liver tissues were collected from adult zebrafish 24 hr after injection with either corn oil or 2 g/kg BaP. Whole larvae were collected at 10 days post-fertilization (dpf) after being exposed to either 0.5% v/v dimethyl sulfoxide (DMSO) or 10 ng/L TCDD. BaP – benzo(a)pyrene; Con – control; NTC – no template control; TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WL – whole larvae.

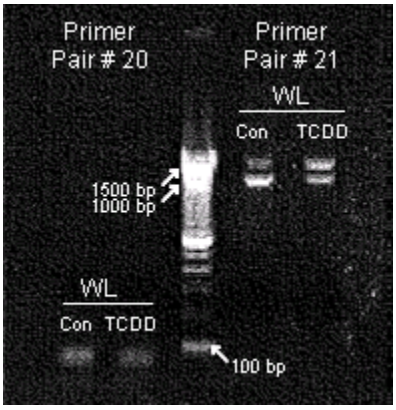


Figure 2.3: Agarose gel to validate primer pairs (#20-21 from Table 2.3) for neuronal nitric oxide synthase (nNOS). DNA ladder gradations are labeled in basepairs (bp). Whole larvae were collected at 10 days post-fertilization (dpf) after being exposed to either 0.5% v/v dimethyl sulfoxide (DMSO) or 10 ng/L TCDD. Con – control; TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WL – whole larvae.

2.2 Determination of Suitable Vehicle for Adult Fish Injections

The initial solvent used in the adult zebrafish exposures was corn oil. Various vegetable oils such as corn oil are commonly used as injection vehicles in toxicology studies because both BaP and TCDD are lipophilic , thus easily incorporate into the oil. The oil was expected to be rapidly absorbed by the fish after intraperitoneal (ip) injection. However, in preliminary experiments, a substantial amount of oil was still present in the abdominal cavity when the peritoneal cavity was opened during dissection 24 hours after the injection. Since all of the oil was evidently not absorbed, it is very likely that all the BaP and TCDD were also not absorbed. Because of this, it was important to find a different solvent that would ensure adequate toxicant delivery, but not exert toxicity by itself. An experiment comparing 24-hr acute vehicle toxicity after ip injection was performed (Table 2.4). TCDD and BaP were determined to be soluble at suitable concentrations for all vehicle solutions tested in Table 2.4. Based on these results, all further experiments examining effects of acute ip injection in adult zebrafish used solutions prepared in 0.5% (volume injected per weight of fish) DMSO.

2.3 Optimizing Egg Rearing Conditions

Before actual experiments could be performed, exposure conditions and larval rearing conditions had to be optimized. It was important to determine the ideal number of eggs that could be placed in each well of the exposure microplates. Therefore, preliminary experiments were conducted, providing optimization as well as baseline values for larval mortalities and deformities to which all subsequent experiments could be compared.

Five, six, and ten eggs were placed into wells in the same conditions that were to be used in the larval experiments (in Chapter 4) using uncontaminated water and reared until 10 days post-fertilization (dpf). Larvae were reared in 3 ml of dechlorinated system water per well with methylene blue added for the first 2 days. Water was changed every day for

Table 2.4: Adult zebrafish exposure solvent mortalities and evidence of tissue damage around injection site 24 hr after intraperitoneal injection. Statistical analysis was not performed since each mortality trial was only performed once or twice for each solvent. DMSO – dimethyl sulfoxide, µl/g – microliters of solvent injected per gram of fish.

Injection Volume (µl/g)	Solvent(s)	Mortality 24 hrs	Notes
10	Toluene	0/6	Severe tissue necrosis around injection site
10	1:4 Toluene:DMSO	4/6	High mortality
10	2:3 Toluene:DMSO	3/6	No initial tissue damage High mortality
5	2:3 Toluene:DMSO	0/6	No observable deleterious effects
10	DMSO	0/6	Tissue necrosis around injection site
5	DMSO	0/6	No observable deleterious effects

Table 2.5: Baseline (untreated) mortality rates at 5 and 10 days post-fertilization (dpf) and overall deformity rates of developing zebrafish incubated at different densities in 12-well microplates (mean ± SEM). n=12-65 wells/group

Number of eggs per well	% Mortality (5 dpf)	% Mortality (10 dpf)	% Deformities (10 dpf)
5	5.2 ± 1.3	11.5 ± 2.1	3.8 ± 1.1
6	4.0 ± 2.1	9.5 ± 5.6	4.0 ± 2.1
10	8.0 ± 1.3	37.3 ± 3.9	6.9 ± 1.1

the first four days and then every second day thereafter. Mortality was noted at 5 and 10 dpf and overall deformities were noted for the total 10 days (Table 2.5). Based on these rates, ten eggs per well was found to be too high a density but five and six eggs per well were comparable. Therefore, 6 eggs per well was used in all subsequent experiments.

2.4 Preliminary *In vivo* Cardiac Function Assessment

2.4.1 Introduction

Cardiac function in 10 dpf zebrafish larvae was hypothesized to respond to pharmacological challenges with acetylcholine (ACh) and norepinephrine (NE). All previous toxicological studies in larval zebrafish have examined cardiac function only under unstimulated conditions (Henry et al., 1997; Andreasen et al., 2002a; Antkiewicz et al., 2005; Carney et al., 2006; Yamauchi et al., 2006). However, at least once basic biological study in developing zebrafish has utilized pharmacological tools to assess larval cardiovascular function (Park et al., 2000). Therefore, the goal of these preliminary experiments was to first assess how the conditions used for immobilizing larval zebrafish affected heart function under unstimulated conditions after developmental exposure to AhR ligands such as BaP and TCDD. Second, the ability of heart rate in normal larvae to respond to exogenously applied norepinephrine and acetylcholine was also examined.

2.4.2 *In vivo* cardiac function measurements

The larvae were prepared in agarose according to methods described by Fritsche et al., (2000). Briefly, zebrafish at 10 dpf were anesthetized in 50 mg/L ethyl 3-aminobenzoate methanesulfonate (MS-222), a common fish and amphibian anesthetic which functions by blocking sodium channels (Wang et al., 1994). The anesthetized larvae were embedded in a lateral position using low melt agarose dissolved in dechlorinated water with 50 mg/L MS-222. The agarose was covered with 1 ml aerated dechlorinated water and fish imaged with an Olympus transilluminated upright microscope equipped

connected to a Canon HV20 high definition digital camcorder. Larvae were imaged using brightfield illumination at 200x magnification. The heart rate was imaged by recording near the trunk of the larvae. Heart rate in beats per minute (bpm) was calculated by counting the number of heart beats in 10 seconds and multiplying by 6.

The effects of the conditions used for immobilization on heart rate were assessed over a time period of 10 minutes. The fish were embedded in agarose as described above and the beating heart was recorded for 10 minutes. Heart rates between treatment groups were assessed at each time point using a repeated measures ANOVA.

Pharmacological challenges were carried out by adding 100 µl of either water (for baseline measurements), acetylcholine (1 and 10 mM), or norepinephrine (1 and 10 mM) to the water on top of the agarose. Before addition of drug, a baseline recording was taken after addition of water and heart rate monitored for 1.5 minutes. Then, acetylcholine was added to produce a final concentration of 1 mM and effects monitored for 1.5 minutes. Finally, acetylcholine was added again to produce 10 mM and heart rate monitored for an additional 1.5 minutes. Larvae were euthanized at this point and new larvae used to examine effects of norepinephrine in a similar manner.

2.4.3 Effects seen on *in vivo* cardiac function

The time course experiment indicated that there were no significant changes in unstimulated heart rate in the zebrafish larvae from each treatment group at 10 dpf over the 10 minute observation period (Figure 2.4; $p>0.05$ for time and treatment in repeated measures ANOVA). There was also no difference observed between groups of larvae exposed developmentally to AhR ligands such as BaP and TCDD (Figure 2.4). In normal larvae, no significant change occurred after the addition of either pharmacological agent ACh or NE at 10 dpf (Figure 2.5).

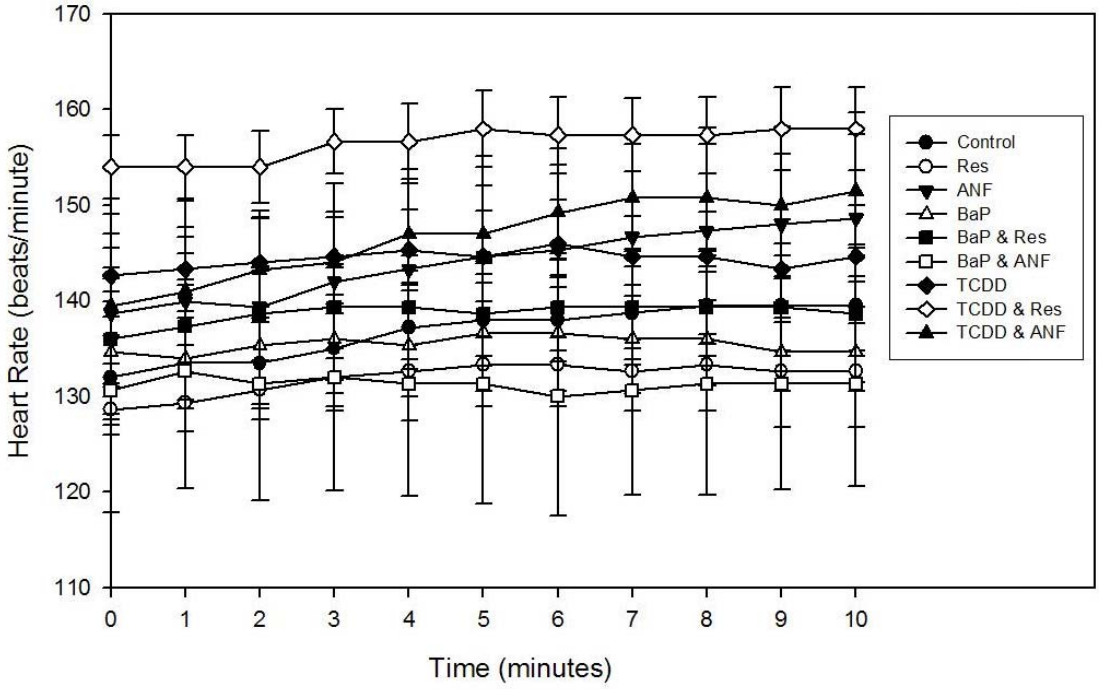


Figure 2.4: Time course of heart rates in larval zebrafish at 10 days post-fertilization (dpf) embedded in agarose from 0 to 10 minutes. Results are mean ± SEM. Larvae were exposed from fertilization until 4 dpf to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with antagonists resveratrol (Res; 300 µg/L) and α-naphthoflavone (ANF; 100 µg/L). Data were analyzed using repeated measures ANOVAs, but no significant differences were detected with either time or treatment. n=8-9 fish per treatment group.

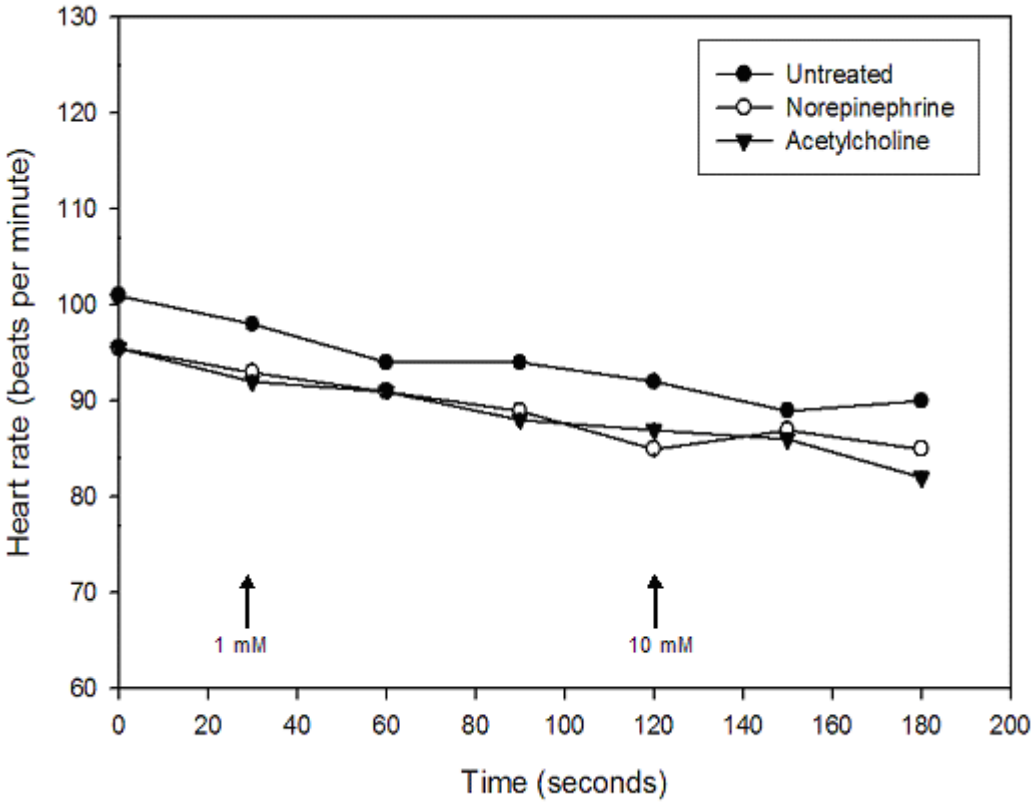


Figure 2.5: Heart rates (beats per minute) of normal larval zebrafish at 10 days post-fertilization after treatment with 1 mM and 10 mM norepinephrine or acetylcholine. Results are mean values. Equivalent volumes of dechlorinated system water were added to the untreated fish instead of drug solution. Arrows indicate addition of pharmacological agents at specified concentrations. n=2 fish per treatment group.

2.4.4 Methodological challenges with *in vivo* analysis of cardiac function

It was apparent that the unstimulated heart rate did not significantly change over time and the method of immobilization was not lethal. There also appeared to be no need for a stabilization period since the heart rate was fairly constant, therefore heart rate was measured immediately from the time of embedding for at least 2 minutes for all experiments in Chapter 4.

In contrast, the attempt to establish methods to challenge the larval zebrafish heart with drugs was a failure. There are a number of reasons that may have led to the inability to see drug-induced changes in heart rate using this method of measuring *in vivo* larval cardiac function. The first reason is that the agarose may have been too thick and the drug did not permeate through to the fish. The second reason is that the drugs permeated the agarose but were not absorbed in sufficient amounts by the larvae to produce an effect. The third reason is that the drugs may actually have been oxidized or metabolized before they were able to cause an effect in the fish. Therefore, all subsequent experiments measured only *in vivo* larval cardiac function under unstimulated conditions. Previous successful experiments using pharmacological tools to manipulate cardiovascular function in agarose-embedded larval zebrafish have used microinjection techniques, which appear to be necessary for success with this technique (Fritsche et al., 2000).

3.0 Hepatic and Vascular CYP and COX mRNA Expression in Adult Zebrafish Following Exposure to Benzo(a)pyrene and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) are widespread environmental contaminants with increasing concentrations being found in aquatic areas near expanding urban centers or through global transport (Van Metre et al., 2000; Borghesi et al., 2008). Dioxin is both potent and persistent in the environment, causing a wide range of toxicities, including immunotoxicity, cardiotoxicity, and reproductive and developmental toxicity (Shertzer et al., 2004). Benzo(a)pyrene (BaP), a member of the PAH family, is not as persistent as TCDD and is rapidly metabolized into often unstable byproducts, such as epoxides, which have mutagenic and cytotoxic effects (Miller and Ramos, 2001). It is widely accepted that these compounds elicit toxic effects at least in part by activating the aryl hydrocarbon receptor (AhR) (Denison and Nagy, 2003). While early life stages of fish species are exceedingly susceptible to PAH and TCDD toxicity (Peterson et al., 1993) with the cardiac and vascular systems being important targets (Henry et al., 1997; Dong et al., 2002), acute TCDD and PAH exposure has been reported to be relatively non-toxic in adult fish (Zodrow et al., 2004; Wang et al., 2008). In contrast adult AhR agonist exposure in mammals is increasingly associated with deleterious effects on the vasculature with the endothelium being the primary target of these toxicants (Kopf et al., 2008; Oesterling et al., 2008). Thus, the acute vascular effects of AhR agonists are unknown in fish, but warrant investigation beginning with an examination of how important vascular and AhR-regulated gene expression is altered.

In order to investigate whether AhR mediates toxicological responses, a pharmacological approach using receptor antagonists could be used. Although resveratrol

has been shown to antagonize AhR-mediated effects on CYP1A induction (Aluru and Vijayan, 2006), there have been no mechanistic studies involving this compound, therefore, it is unclear how it acts as an antagonist. Alpha-naphthoflavone, on the other hand, has been shown to act as an antagonist via competitive receptor binding (Merchant et al., 1993) and via CYP1A substrate binding (Tassaneeyakul et al., 1993; Schaeffner et al., 2005), although the latter method of antagonism should not affect mRNA induction. Therefore, these two antagonists will be used in combination with TCDD and BaP.

AhR activation has been shown to affect the transcription of cytochrome P450 (CYP) isozymes 1A and 1B1 (Nebert et al., 2000), as well as CYP1C1, a gene which appears to be exclusive to fish species (Godard et al., 2005; Wang et al., 2006; Jönsson et al., 2007a, b). Hepatic CYP1A is a common biomarker of AhR agonist exposure, but CYP1A expression significantly increases in multiple tissues after AhR agonist exposure including vascular endothelial cells in both mammalian and piscine species (Kerzee and Ramos, 2001; Dong et al., 2002; Teraoka et al., 2002). However, CYP1A expression increases to a much lesser extent in vascular smooth muscle (Kerzee and Ramos, 2001). Instead, smooth muscle CYP1B1 expression has been reported to be increased after AhR agonist exposure in the murine model (Kerzee and Ramos, 2001). In fish, CYP1B1 has been reported to have high constitutive expression in gill tissue, but may also increase after agonist exposure in liver, intestine, and gonad tissues (Willett et al., 2006). More recently, CYP1C1 expression was shown to be significantly increased by BaP and PCB-126 in heart, liver, and gill tissues of adult fish (Wang et al., 2006; Jönsson et al., 2007a, b). Similarly, expression of CYP1C1 and CYP1C2 were increased in larval zebrafish in an AhR-dependent manner in most tissues (Godard et al., 2005; Jönsson et al., 2007b). Thus, CYP1A expression appears consistently upregulated after AhR agonist exposure in most tissues previously examined except vascular smooth muscle, suggesting that tissue differences in AhR-mediated changes in gene expression need to be clarified. A goal of the current study was to examine gene expression changes specifically in vascular tissue because of the large number of vascular abnormalities after developmental AhR agonist exposure in fish (Dong et al., 2002; Teraoka et al., 2002; Billiard et al., 2006) and the increasing focus on AhR agonist effects in adult mammalian vascular tissue (Miller and

Ramos, 2001; Jang et al., 2007; Kopf et al., 2008; Oesterling et al., 2008). Although all highly perfused tissues such as liver contain vascular beds which would contribute to the overall gene expression profile, hepatocyte gene expression will predominate when this tissue is examined. Therefore, the current study compared the better characterized AhR-mediated changes in hepatic gene expression with that in the relatively unknown mesenteric artery.

Cyclooxygenases (COXs), also known as prostaglandin endoperoxide synthases, are involved in the conversion of arachidonic acid to prostaglandins. Zebrafish have three functional and constitutively expressed COX genes: COX-1, COX-2a, and COX-2b (Grosser et al., 2002; Ishikawa et al., 2007). The COX-1 transcripts have been identified in various adult zebrafish tissues, including gut, liver, gonad, heart and skeletal muscle while COX-2a expression was seen in the gills, gut, testis, heart and brain (Grosser et al., 2002; Ishikawa et al., 2007). Some COX-2b expression has been found in high levels in the gill, heart, and ovary, while lower expression levels were found in the kidney, gut, testis, eyes, and brain (Ishikawa et al., 2007). Knockdown of COX-1 in developing zebrafish has been shown to result in defects in vascular tube formation indicating a critical role in normal vascular development (Cha et al., 2005) while knockdown of COX-2a did not cause a discernable developmental phenotype (Grosser et al., 2002; Cha et al., 2005). Recently, COX-2a gene expression was found to be necessary and sufficient for mediating developmental TCDD toxicity, yet TCDD failed to increase COX-2a expression in whole larvae (Teraoka et al., 2009). In contrast, the effect of AhR agonists on COX-1 or COX-2b expression are not known in developing fish, while TCDD effects on all COX isoforms in adult fish are unknown. Because of the importance of prostaglandins to the function of the adult fish cardiovascular system (Farrell and Johansen, 1995), the effect of AhR agonists on these genes was a focus of this study in adult fish.

Therefore, it was hypothesized that acute AhR agonist exposure in adult fish would cause changes in gene expression that would differ between vascular and hepatic tissues. It was also hypothesized that the magnitude of CYP mRNA expression increase induced by AhR agonists would be greater in hepatic tissue while COX mRNA expression would increase to a greater extent in vascular tissue. In order to investigate these hypotheses,

mRNA expression of CYP and COX isozymes were quantified in adult zebrafish liver and mesenteric artery 24-h after exposure to TCDD or BaP alone or in combination with the purported AhR antagonists resveratrol (Res) and α-naphthoflavone (ANF).

3.2 Materials and methods

3.2.1. Chemicals

Benzo(a)pyrene (B1760), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (10 μg/ml in toluene; 48599), resveratrol (Res; R5010), α-naphthoflavone (ANF; N5757), dimethyl sulfoxide (DMSO; D5879) and ethyl 3-aminobenzoate methanesulfonate (MS-222; E10521) were all purchased from Sigma–Aldrich (Oakville, Ontario, Canada). The toluene in the TCDD stock was evaporated under nitrogen and then a dilute TCDD stock (2 μg/ml) was reconstituted in DMSO. All other chemical stocks for injection were dissolved directly in DMSO.

3.2.2 Fish maintenance

All fish housing and experimental procedures were approved by the Animal Research Ethics Board at the University of Saskatchewan in accordance with guidelines of the Canadian Council on Animal Care. Adult zebrafish were purchased from a local pet store and acclimated for 4 weeks in a static tank with particulate and charcoal filters, as well as ammonia biofilters prior to treatment. Fish were maintained at 28.5 °C with a photoperiod of 14 h light and 10 h dark. They were kept on a diet of Nutrafin Max Complete Flake Food for Tropical Fish alternated with Hikari Bio-Pure frozen bloodworms. Food was withheld for 24 h prior to and after injection.

3.2.3 Exposure

Adult fish (0.5 – 1 g) were anaesthetized with 120 mg/L MS-222 until they were unresponsive to a tail pinch test. Fish (18 per treatment group) were injected with 5 μl of

test solution per gram of fish using an ultrafine needle on a 25-µl Hamilton syringe in the intraperitoneal cavity. Since arteries from multiple fish had to be pooled for a single sample, feasibility required a limited number of treatments to be examined. Therefore, single, acutely sublethal doses of AhR agonists and antagonists were chosen based on previous reports. Fish received a single injection of the vehicle, DMSO, or the following compounds alone or in combination: BaP (1 mg/kg; Wang et al., 2008), TCDD (20 µg/kg; Zodrow and Tanguay, 2003; Zodrow et al., 2004), Res (10 mg/kg–upper solubility limit of Res in vehicle; Aluru and Vijayan, 2006; Canistro et al., 2009) or ANF (50 mg/kg; Aluru and Vijayan, 2004). Following injections, fish were recovered individually in an aerated beaker before being transferred to a static exposure tank where all fish from a given treatment group were housed. No mortalities occurred in any of the treatment groups. Fish were euthanized 24 h after injection with an overdose of MS-222. A 24-h post-injection time was chosen in this study because it has been previously reported as the earliest near-maximum point in mRNA expression after injection of AhR agonist in adult fish tissue (Chung-Davidson et al., 2004). Liver and mesenteric artery were removed, with a single liver being sufficient for one sample, while mesenteric arteries from 3 fish were pooled for each sample.

3.2.4 Real-time rt-PCR

Real-time rt-PCR was carried out to examine expression levels of CYP1A, CYP1B1, CYP1C1, CYP1C2, COX-1, COX-2a, and COX-2b. Total RNA was extracted from liver and artery samples with Trizol (Invitrogen, Burlington, Ontario, Canada) according to manufacturer's specifications, then quantity (A_{260}) and quality ($A_{260:280}$; purity > 1.8 for all acceptable samples) assessed using a NanoDrop apparatus and software (Thermo Fisher Scientific). cDNA templates were synthesized from total RNA with Superscript II reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time rt-PCR analysis was performed in a Stratagene MX3005P QPCR System (VWR, Mississauga, Ontario, Canada) using SYBR Green qPCR Supermix (VWR) with a reaction volume of 25 µl. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) of zebrafish was used as a

housekeeping gene (internal control) and its expression was determined for every PCR run for every sample. Primers were designed in this laboratory based on GenBank zebrafish sequences using Geneious software (Biomatters Ltd) for multiple sequence alignments, DINAMelt (http://dinamelt.bioinfo.rpi.edu/quikfold,php) to predict melting profiles and Amplify (Bill Engels, University of Wisconsin) to run PCR simulations to predict product fragment size. The primer pairs for the genes of interest can be found with their accession numbers in Table 3.1. All primers were confirmed to produce only one gene product based on a single peak in the melting curve and a single band of the predicted size detected on agarose gels in preliminary studies. PCR efficiencies were determined to be ≥100% for all genes examined in the current study.

3.2.5 Data and statistics

All expression data for genes of interest were expressed as ΔΔCT values first using expression of the housekeeping gene (G3PDH) as an internal correction for expression of all genes of interest, followed by correction for control group expression of the gene of interest to calculate fold-change with treatment (Livak and Schmittgen, 2001). Results are presented as mean ± standard error of the mean (SEM). All data was log transformed to achieve normality (Shapiro–Wilk's test) and homogeneity of variance (Levene's test). Significant differences among groups were initially determined using a 2-way analysis of variance (ANOVA) with treatment and tissues as factors. However, there were significant interactions between these two factors. Therefore, the analyses were split and separate 1-way ANOVAs were run for each tissue, followed by Tukey's posteriori tests as appropriate with $p < 0.05$ considered to be significantly different.

3.3 Results

3.3.1 Effects of AhR agonists on mRNA expression

Dioxin significantly increased ($p < 0.05$ in Tukey's test after 1-way ANOVA) expression of hepatic CYP1A (104.8±21.1 fold-increase above control), CYP1C1 (12.0±2.1), and COX-2b (2.2±0.3) mRNA as well as mesenteric artery CYP1A (120.6±22.9), CYP1B1 (5.2±1.2), CYP1C1 (28.7±6.3), CYP1C2 (6.7±0.9), and COX-1 (2.5±0.3) mRNA (Figure 3.1). Benzo(a)pyrene significantly increased expression of COX-1 (3.2±0.7) and COX-2b (1.6±0.1) in the liver, as well as expression of CYP1A (2.1±0.3), CYP1B1 (4.0±0.3), CYP1C1 (5.1±0.5), CYP1C2 (4.7±0.6), and COX-1 (2.3±0.3) in the mesenteric arteries (Figure 3.1).

3.3.2 Effects of AhR antagonists on baseline mRNA expression

Resveratrol had no significant effect on baseline mRNA expression of any of the genes examined in both hepatic and vascular tissue (Figure 3.2). On the other hand, ANF significantly increased CYP1A expression in both hepatic (61.8±8.5) and vascular tissue (11.1±1.5; Figure 3.2). Paradoxically, ANF significantly decreased CYP1B1 expression (0.3±0.04) in hepatic tissue, but increased CYP1B1 (5.9±0.3) in mesenteric artery (Figure 3.2). Alpha-naphthoflavone alone also increased COX-1 expression in hepatic (3.5±0.7), but not mesenteric artery (Figure 3.2). Furthermore, ANF significantly increased mesenteric artery expression of CYP1C1 (4.6±0.6), CYP1C2 (6.6±1.4), COX-2a (3.5±0.8), and COX-2b (6.0±1.3; Figure 3.2).

3.3.3 Effects of AhR antagonists on AhR agonist-induced mRNA expression

Resveratrol had no significant effect on BaP-induced changes in mRNA expression of any of the genes examined in both hepatic and vascular tissues, while it did reduce TCDD-induced CYP1C2 expression in the artery by 52% (Figure 3.3). In contrast, ANF significantly antagonized the effects of BaP-induced increases in hepatic CYP1B1 and

Table 3.1: cDNA target genes, GenBank accession numbers, and primers used for real-time rt-PCR.

Gene	Primer	Sequence (5' – 3')	Accession Number
CYP1A	*Forward*	TGCCGATTTCATCCCTTTCC	NM_131879
	Reverse	TTCGGTCTTCGCAGTGGTTGA	
CYP1B1	*Forward*	AGACCTGACCACCAACGTGC	NM_001013267
	Reverse	ATGTTCCTACCAAGCTGTCC	
CYP1C1	*Forward*	AGCGGAGGTGCATTGGTGAG	NM_001020610
	Reverse	ATGCCGGTGAAACCAAGCCA	
CYP1C2	*Forward*	GATAGCATGGCGCAGTCGGA	NM_001114849
	Reverse	TTCCAACAAGCGGCCAAGCA	
COX-1	*Forward*	GGAGATTCTCTTGATCGCCA	NM_017043
	Reverse	CTCACGAAGCCACAAGGTAG	
COX-2a	*Forward*	CAGGTTGTGGATGGTGAGGT	NM_153657
	Reverse	CACCAATCAGGATGAGACGA	
COX-2b	*Forward*	GCTCAAGTTTGATCCCGAAC	NM_001025504
	Reverse	GCTGTTGACGCCATAATCTGT	
G3PDH	*Forward*	AGCACTGTTCATGCCATCAC	NM_001115114
	Reverse	TACTTTGCCTACAGCCTTGG	

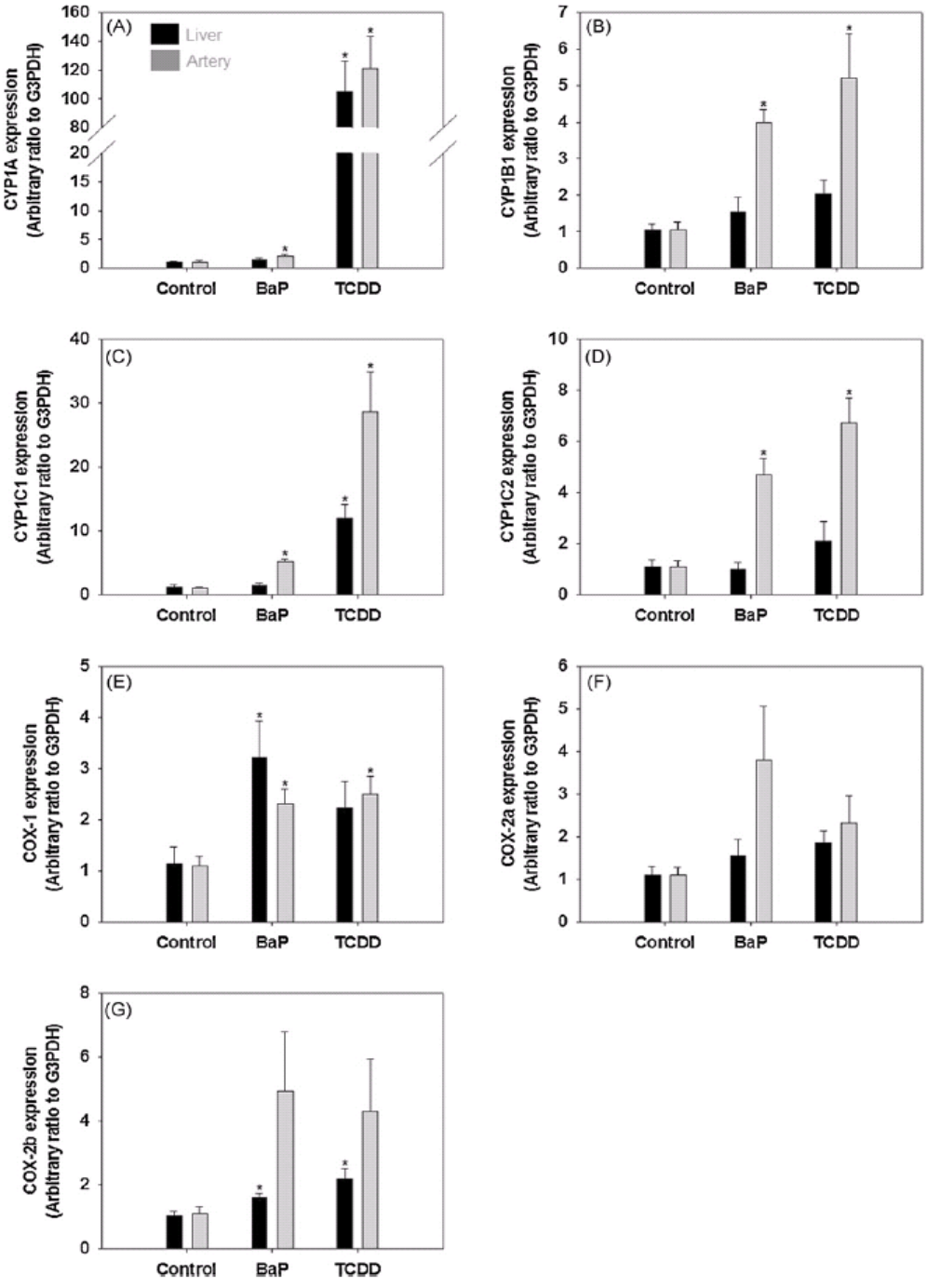


Figure 3.1: (A) CYP1A, (B) CYP1B1, (C) CYP1C1, (D) CYP1C2, (E) COX-1, (F) COX-2a, and (G) COX-2b mRNA expression (fold change relative to control levels) in adult zebrafish liver (black bars) and mesenteric artery (grey bars) tissues after 24-h exposure to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP) and dioxin (TCDD). Results are mean ± SEM. Asterisks indicate statistically significant ($p < 0.05$, n = 4–6/treatment group) differences from control treatment values after 1-way ANOVA and Tukey's posteriori test.

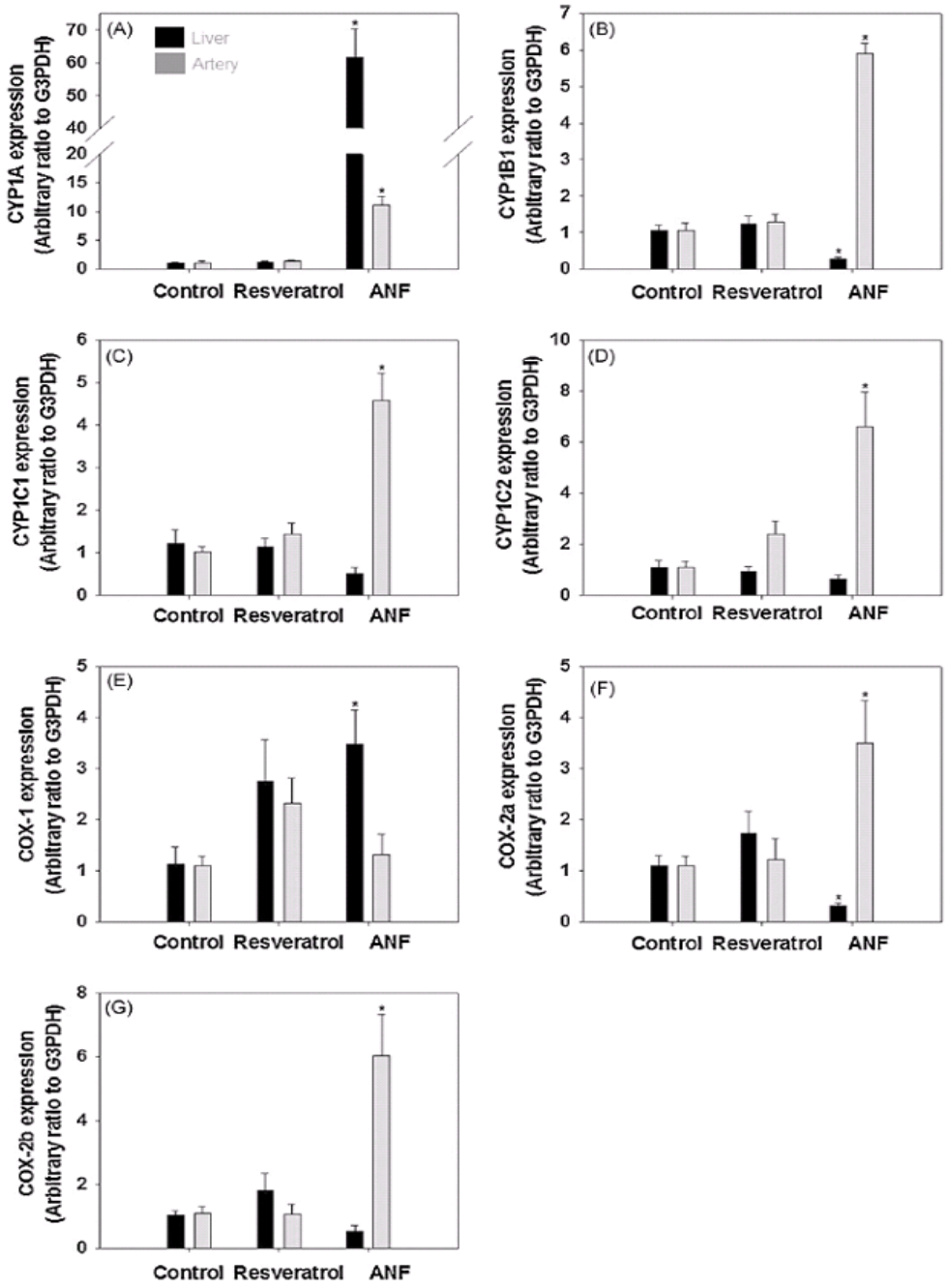


Figure 3.2: (A) CYP1A, (B) CYP1B1, (C) CYP1C1, (D) CYP1C2, (E) COX-1, (F) COX-2a, and (G) COX-2b mRNA expression (fold change relative to control levels) in adult zebrafish liver (black bars) and mesenteric artery (grey bars) tissues after 24-h exposure to aryl hydrocarbon receptor antagonists resveratrol (Res) and α-naphthoflavone (ANF). Results are mean ± SEM.Asterisks indicate statistically significant ($p < 0.05$, $n = 4–6$/treatment group) differences from control treatment values after 1-way ANOVA and Tukey's posteriori test.

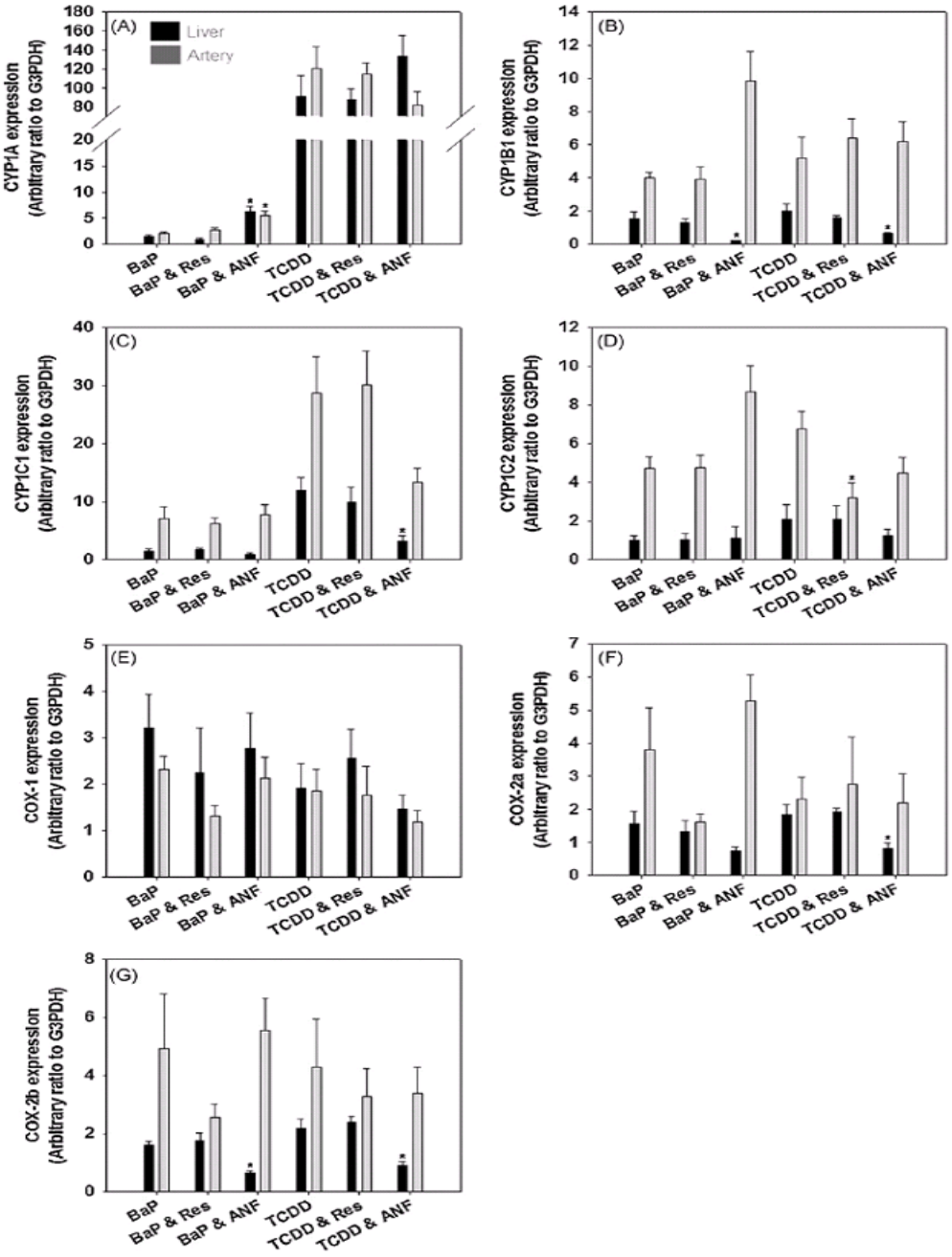


Figure 3.3: (A) CYP1A, (B) CYP1B1, (C) CYP1C1, (D) CYP1C2, (E) COX-1, (F) COX-2a, and (G) COX-2b mRNA expression (fold change relative to control levels) in adult zebrafish liver (black bars) and mesenteric artery (grey bars) tissues after 24-h exposure to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP) and dioxin (TCDD) in combination with antagonists resveratrol (Res) and α-naphthoflavone (ANF). Results are mean ± SEM. Asterisks indicate statistically significant ($p < 0.05$, n = 4–6/treatment group) differences from agonist alone treatment values after 1-way ANOVA and Tukey's posteriori test.

COX-2b expression (reduced by 85% and 60%, respectively, compared to BaP alone; Fig. 3). Alpha-naphthoflavone also antagonized the effects of TCDD on hepatic CYP1B1, CYP1C1, COX-2a, and COX-2b expression (reduced by 69%, 74%, 56%, and 58%, respectively compared to TCDD alone). However, in mesenteric artery, ANF had no significant antagonistic effects on BaP- or TCDD-induced changes in mRNA expression (Figure 3.3).

3.4 Discussion

The most intriguing finding of this study was that although ANF exhibited some antagonist action in adult zebrafish liver against genes other than CYP1A, it acted as an AhR agonist in arterial tissues from the same fish. While there have been numerous studies examining the effects of developmental AhR agonist exposure, few studies have examined effects of AhR agonist exposure during the adult stage in fish. Of those studies examining adult AhR agonist exposure, effects on CYP gene expression or enzyme activity have been reported only in fish liver and brain, although several mammalian studies have reported effects in vascular endothelial cells. In contrast, there are no previous reports of adult AhR agonist exposure effects in fish blood vessels. This study demonstrates that 24-h sublethal AhR agonist exposure elicits quite different expression patterns in fish mesenteric artery compared to liver. Finally, we have shown that AhR stimulation alters expression of the inducible zebrafish cyclooxygenase genes COX-2a and COX-2b, as well as the constitutive COX-1, all of which are important in adult fish cardiovascular function.

Dioxin is an extremely potent AhR agonist that exerts more developmental toxicity and induces higher larval expression levels of CYP1A compared to the weaker AhR agonist, BaP (Ortiz-Delgado and Sarasquete, 2004), in agreement with the results of the current study in adult fish. Previous studies using aqueous exposures of the AhR agonist, BaP, have reported hepatic and vascular alterations in CYP1A or CYP1C1 expression (Ortiz-Delgado and Sarasquete, 2004; Wang et al., 2006). The current study failed to reproduce these effects of BaP on hepatic CYP expression, despite an exposure method (ip

injection) that guaranteed delivery of a high dose. However, since CYP enzyme expression was increased in mesenteric artery from the same fish, the BaP dose was successfully delivered in the current study. Instead, differences in time to respond to BaP or tissue sensitivity may be an explanation for failure to see changes in hepatic CYP expression after BaP injection. Several studies using a single, sub-lethal dose of TCDD higher than that used in the current study reported that CYP1A increased in multiple tissues of adult fish, including kidney, liver, gill, heart, gastrointestinal tract, and caudal fin, as well as in regenerating tissue (Zodrow et al., 2004). The current study, using a lower dose of TCDD, demonstrated that not just CYP1A expression was altered, but also CYP1B1, CYP1C1, CYP1C2, COX-1, and COX-2b.

Differences in gene expression after TCDD versus BaP exposure in adult fish may be due to the ability of the fish to metabolize the parent compounds. Dioxin is not metabolized in the body and is, therefore, highly persistent and able to bioaccumulate in food chains (Piskorska-Pliszczynska et al., 1986). Since it is not broken down, it remains active in the system, constantly binding and activating the AhR, leading to the high expression levels observed in the current study. Benzo(a)pyrene, on the other hand, is easily metabolized and actually initiates its own metabolism by inducing CYP1A (Miller and Ramos, 2001). Therefore, one explanation for the relative lack of BaP effect on hepatic gene expression may be that the BaP, at the dose used in the current study, was rapidly metabolized in the adult fish, preventing sufficient AhR activation to change hepatic mRNA levels. However, since BaP was able to induce a small increase in arterial CYP1A gene expression, sufficient BaP must have reached this tissue to cause AhR activation. Previous studies have reported minimal or no induction of hepatic CYP1A protein and ethoxyresorufin-o-deethylase (EROD) activity after repeated ip injection with the same dose of BaP as the current study in other fish species (Wang et al., 2008). Therefore, mesenteric artery AhR may be more sensitive or responds faster to BaP than hepatic AhR in zebrafish at the time and dose examined in the current study. However, a full dose-response and time-course experiment correlating BaP- to TCDD-induced effects on gene expression is necessary to confirm differences in tissue sensitivity to these compounds.

Resveratrol has been reported to be an effective AhR antagonist in rainbow trout (*Oncorhynchus mykiss*) hepatocyte cultures, reducing CYP1A mRNA after 24 h of exposure, but was also shown to act as a weak AhR agonist at doses lower than 10^{-8} M the same cultures (Aluru and Vijayan, 2006). Despite the successful use of resveratrol to abolish a variety of AhR induced effects (Casper et al., 1999) or to inhibit basal activity of several CYP enzymes in mice (Canistro et al., 2009), the efficacy of resveratrol as an AhR antagonist has yielded conflicting results when given *in vivo* rather than used in cell cultures or purified systems (Canistro et al., 2009). Since the current study failed to see any agonistic or antagonistic effects of resveratrol on AhR after ip injection in zebrafish, the *in vivo* efficacy of resveratrol is questionable. In fact, resveratrol is now known to interfere with a multitude of pathways, including COX-2 (Degner et al., 2009). Resveratrol has been shown to have an extremely low bioavailability in humans when given orally, therefore, the dose used, despite being a high one, may have been inadequate to produce systemic effects (Brown et al., 2009). However, since only one dose (albeit a high dose) and time point was examined in the current study, antagonistic effects may have been missed and require further investigation.

Alpha-naphthoflavone (ANF) has been used to effectively antagonize AhR-mediated effects such as teratogenicity and apoptosis of neuronal cells in mammals (Jang et al., 2007; Kajta et al., 2009), as well as embryotoxicity and EROD activity in fish (Dong et al., 2002; Jos et al., 2007). However, ANF has been reported to act as either a weak agonist or an antagonist in mammalian cell lines, depending on the concentration used (Gasiewicz and Rucci, 1991). This agrees with the results of the current study in zebrafish and other studies in isolated rainbow trout hepatocytes (Aluru and Vijayan, 2004). Furthermore, ANF in combination with the AhR agonist beta-naphthoflavone (BNF) increases not only hepatic CYP1A protein expression (Aluru and Vijayan, 2004), but also promotes synergistic induction of CYP1A, CYP1B1, and CYP1C1 expression as well as synergistic increases in developmental deformities in larval fish (Billiard et al., 2006; Timme-Laragy et al., 2007). The novel observation in the current study that ANF by itself was able to increase expression of multiple CYP enzymes in vascular, but not hepatic tissue from the same fish may suggest that vascular tissue is more sensitive or responds faster to AhR agonists.

However, this possible difference in tissue sensitivity cannot be determined unless full dose-response and time-course of ANF effects are examined in future studies. Alternatively, although the current study did not provide any mechanistic evidence, ANF may produce tissue specific responses because it also activates AhR-independent mechanisms in mesenteric artery.

The major focus of this study was to determine whether acute exposure to AhR agonists in adult fish causes potentially physiologically-relevant alterations in vascular gene expression. Therefore, it stands to reason that genes other than CYP1A, such as COXs which play key roles in adult fish cardiovascular function, may be physiologically-important. Our findings that vascular CYP1C1 mRNA expression was increased in a similar pattern, but not to the same magnitude, as CYP1A indicate that CYP1C1 could play a role in AhR agonist toxicity. The results of the current study confirmed the presence of COX-1 in hepatic tissues (Grosser et al., 2002; Ishikawa et al., 2007). However, control group COX-2a and COX-2b transcripts were also detected with only COX-2b expression being increased by AhR agonist exposure. This could signify a more constitutive role of COX-2 isozymes in the liver, rather than the more commonly accepted inflammatory-related role (Degner et al., 2009). In agreement with the current study, TCDD was reported to induce COX-2 expression in mouse hepatoma cells (Puga et al., 1997). Since the response pattern observed in the arteries was different and involved more genes compared to the liver, the vasculature should be further investigated as a target for AhR-mediated effects in adult fish. Furthermore, as BaP failed to induce hepatic CYP1A expression while still altering expression of other genes in vascular tissue, future studies should not rely solely on CYP1A as an indicator of AhR activity.

Cyclooxygenase enzymes are reported to be required for normal blood vessel development in zebrafish (Cha et al., 2005) and for prostaglandin production which is of potential importance in fish vascular relaxation (Farrell and Johansen, 1995). Recent findings have indicated that COX-2a expression is necessary for TCDD-induced circulation failure in mesencephalic vein, but not arteries located in the trunk of the developing zebrafish (Teraoka et al., 2009). However, the same previous study failed to detect any increase in COX-2 isozymes by TCDD when expression was determined in whole larvae

(Teraoka et al., 2009). The current study also failed to detect significant increases in COX-2a or COX-2b expression despite similar or larger magnitudes of increase compared to that observed in liver. Future studies must determine whether a Type II statistical error was made in the current study and clarify whether COX-2a and COX-2b increase in vascular tissue after AhR agonist exposure in adult fish. Therefore, the AhR agonist-induced alterations in COX-1 expression observed in the current study could interfere with vascular function similar to that reported after TCDD exposure in mammals (Kopf et al., 2008). Clearly the potential for physiologically-relevant effects would be even higher if COX-2 isozymes are also affected. This study utilized whole mesenteric artery, making it unclear whether the expression changes are in the endothelium, smooth muscle, adventitia, or a combination. However, the increased vascular CYP1B1 expression is likely to be within the smooth muscle cells similar to that reported in mammals (Kerzee and Ramos, 2001). Regardless of which vascular cells are responsible for the increased expression of CYP or COX isozyme mRNA, clearly vascular tissue such as mesenteric artery is a responsive target for AhR agonist action in adult fish. Concurrent studies from this laboratory have found that vasorelaxant responses to exogenous prostaglandin E2 were unaltered in dorsal aorta after acute BaP exposure in rainbow trout, while responses to other vasorelaxing agents such as nitric oxide and hydrogen sulfide donors were altered (Goertzen and Weber, 2008). However, whether the increased mRNA expression leads to alterations in COX protein levels, increased endogenous prostaglandin production and ultimately in biologically significant effects on cardiovascular function in fish needs to be confirmed in future experiments.

In conclusion, neither resveratrol nor ANF appears to be suitable for use as AhR antagonists. Furthermore, there are important differences in acute responses in gene expression to both AhR agonists and antagonists between liver and arteries in adult zebrafish. The vascular-specific changes in gene expression will be linked to future studies examining alterations in cardiovascular function produced by acute AhR agonist exposure in adult fish. If confirmed in future studies, vascular responses to AhR agonists could negatively affect the ability of fish to regulate their blood pressure, repair blood vessels, impair the ability to swim, and ultimately reduce survivability of an individual fish.

4.0 Phenotypic Anchoring of Gene Expression After Developmental Exposure to Aryl Hydrocarbon Receptor Ligands in Zebrafish

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BaP) and halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are widespread environmental contaminants (Van Metre et al., 2000; Borghesi et al., 2008) that cause a variety of reproductive, developmental, and cardiovascular toxicities (Shertzer et al., 2004; King Heiden et al., 2009). Activation of the aryl hydrocarbon receptor (AhR) is critical for these compounds to elicit many of their developmental toxicities (Denison and Nagy, 2003). Ligand binding to AhR stimulates translocation and dimerization of the receptor with the AhR nuclear translocator, resulting in transcriptional activation of the Ah gene battery (Nebert et al., 2000), including cytochrome P450 monooxygenase (CYP) enzymes, most notably CYP1A, CYP1B1, CYP1C1, and CYP1C2 (Nebert et al., 2000; Godard et al., 2005; Wang et al., 2006; Jönsson et al., 2007a, b). Using knock down approaches with antisense morpholinos in zebrafish (*Danio rerio*), AHR2 and ARNT1 appear to be necessary for TCDD-mediated developmental deformities (Antkiewicz et al., 2006; Prasch et al., 2006). Increased expression of CYP1A precedes cardiovascular toxicity (Cantrell et al., 1996; Guiney et al., 2000) and CYP1A knockdown may prevent pericardial edema and circulation failure (Teraoka et al., 2003) in TCDD-exposed fish. However, the necessity for CYP1A in TCDD-induced toxicity is equivocal since CYP1A knockdown in another study failed to prevent TCDD-induced developmental toxicity (Carney et al., 2004). Furthermore, developmental deformities persisted into the F1 generation of zebrafish despite an absence of detectable CYP1A activity (King Heiden et al., 2009). Finally, CYP1A knockdown may actually increase BaP and α-naphthoflavone (ANF)-mediated developmental deformities while CYP1B1 knockdown had no effect in zebrafish (Timme-Laragy et al., 2008). Thus, the role of CYP1A in developmental toxicity

requires clarification. Furthermore, expression of other CYP isoforms, specifically CYP1B1, CYP1C1, and CYP1C2 have been shown to significantly increase in cardiovascular tissue after AhR agonist exposure (Wang et al., 2006; Bugiak and Weber, 2009). Therefore not only should other CYPs be investigated for their role in AhR-mediated developmental toxicity, but other genes are known to be responsive to AhR ligands in cardiovascular tissue (Carney et al., 2004; Bugiak and Weber, 2009) and require further investigation.

Cyclooxygenases (COXs), also known as prostaglandin endoperoxide synthases, are involved in the conversion of arachidonic acid to prostaglandins. Zebrafish have three functional COX genes: COX-1, COX-2a, and COX-2b (Grosser et al., 2002; Ishikawa et al., 2007). Constitutive COX-1 is widely expressed during larval development and is primarily responsible for prostaglandins required for gastrulation and segmentation (Cha et al., 2005, 2006; Pini et al., 2005). In contrast, COX-2 expression is responsible for inflammatory responses and may contribute to vascular tone (Tilley et al., 2001; Pini et al., 2005). A previous study from this laboratory reported that COX-1, COX-2a, and COX-2b mRNA levels are all significantly increased after acute exposure to AhR agonists in adult zebrafish (Bugiak and Weber, 2009). Also, another recent study found that COX-2a was a necessary mediator of TCDD-induced reductions in mesencephalic vein blood flow, but not reductions in blood flow in the trunk of developing zebrafish (Teraoka et al., 2009). Thus, while COX expression is emerging as an important factor in cardiovascular function and development (Farrell and Johansen, 1995; Teraoka et al., 2009), the relationship to AhR activity, the specific COX subtype(s) involved and subsequent phenotypic anchoring require further investigation.

Heart defects induced by AhR agonists, TCDD in particular, include decreased heart rate, decreased size, disruption in valve formation, and alterations in looping (Incardona et al., 2004; Antkiewicz et al., 2005; Mehta et al., 2008). Pericardial edema increases pressure around the heart which may lead to a lack of heart looping (Teraoka et al., 2002). The unlooped heart has a decrease in cardiac output potentially explaining the resulting decrease in a blood flow, a reported indication of vascular toxicity (Henry et al., 1997; Belair et al., 2001; Dong et al., 2002, 2004; Teraoka et al., 2002). Several studies

have noted cardiac changes prior to manifestation of circulatory effects (Teraoka et al., 2002; Antkiewicz et al., 2005; Mehta et al., 2008), suggesting that cardiac changes are the primary developmental lesion and circulatory failure is secondary. If so, then AhR-mediated changes in gene expression should show similar relationships to cardiac and vascular developmental defects. However, the relationship between AhR-mediated changes in cardiac gene expression and cardiac phenotype is still largely unknown (Handley-Goldstone et al., 2005). Therefore, there is a need for further anchoring of the developmental cardiovascular phenotype with gene expression over a wide variety of conditions.

Whether a primary or secondary target of AhR-mediated developmental toxicity, early vascular development is a sensitive target of AhR-agonist-mediated toxicity as evidenced by regression failure of embryonic vascular structures, vascular permeability, declination of peripheral vascular bed health, and subsequent fin rot (Guiney et al., 1997; Henry et al., 1997; Andreasen et al., 2002a; Dong et al., 2004). There is a critical window of time when developmental circulatory impairment can be induced from AhR agonist exposure which falls between 2 and 4 dpf (Belair et al., 2001). This circulatory failure precedes TCDD-induced mortality (Henry et al., 1997) and reductions in red blood cell perfusion rates in the brain and trunk of developing zebrafish can be detected as early as 48 hours post fertilization, several hours before completion of heart formation (Dong et al., 2002; Teraoka et al., 2002). Previous studies suggest that TCDD does not affect initial development of the vasculature, including size, number, and patterning of blood vessels, and it does not prevent initial blood flow to the trunk (Henry et al., 1997; Belair et al., 2001). However, peripheral vascular bed health and function decline (Henry et al., 1997), while embryonic yolk-associated structures, such as the common cardinal vein and vitelline vasculature, fail to regress in a normal fashion (Hornung et al., 1999; Bello et al., 2004; Goldstone and Stegeman, 2006). Developmental TCDD exposure has been reported to cause DNA damage and subsequent vascular cell death (Cantrell et al., 1996) which may be related to concomitant increases in vascular permeability (Guiney et al., 2000; Dong et al., 2004). In contrast, blood flow is decreased after TCDD exposure and the vascular

endothelial cells appear distinctly sensitive to CYP1A induction (Guiney et al., 1997; Henry et al., 1997; Belair et al., 2001; Dong et al., 2002, 2004).

Although knockdown approaches using antisense morpholino oligonucleotides are perhaps the most common approach used for phenotypic anchoring of AhR-mediated developmental toxicity in larval zebrafish, contradictory results have occurred. Thus, the current study used a different approach for phenotypic anchoring. A variety of AhR ligands with slightly different, but overlapping mechanisms of action were used in order to dissect genes that were important for producing common aspects of a deformed phenotype. It was hypothesized that CYP mRNA expression would be correlated with cardiac defects while COX mRNA expression would be correlated with vascular alterations. The objective of the current study was to first determine effects on CYP and COX gene expression patterns with different AhR ligands in larval zebrafish. Second, this study aimed to correlate these expression patterns with a variety of cardiovascular and developmental endpoints. In order to carry out this objective, mRNA expression of CYP and COX isoforms were quantified in whole larvae 5 and 10 days post-fertilization (dpf) after exposure to TCDD and BaP alone or in combination with the purported AhR antagonists resveratrol (Res) or α-naphthoflavone (ANF).

4.2 Materials and Methods

4.2.1 Chemicals

Benzo(a)pyrene (B1760), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (10 μg/ml in toluene; 48599), resveratrol (R5010), α-naphthoflavone (N5757), dimethyl sulfoxide (DMSO, D5879) and ethyl 3-aminobenzoate methanesulfonate (MS-222; E10521) were all purchased from Sigma-Aldrich (Oakville, ON, Canada). The toluene in the TCDD stock was evaporated under nitrogen and a 2 μg/ml TCDD stock was reconstituted in DMSO. All other chemical stocks for exposures were dissolved directly in DMSO.

4.2.2 Fish Maintenance and Egg Exposures

All fish housing and experimental procedures were approved by the Animal Research Ethics Board at the University of Saskatchewan in accordance with guidelines of the Canadian Council on Animal Care. Adult zebrafish were purchased from a local pet store and acclimated for at least four weeks in a flow-through tank. Fish were maintained at 28.5°C with a photoperiod of 14h light and 10h dark. They were kept on a diet of Nutrafin Max Complete Flake Food for Tropical Fish alternated with Hikari Bio-Pure frozen bloodworms. Eight adult fish (three male, five female) were placed into each static breeding tank, eggs collected the following morning and placed in Petri dishes with system water containing 0.002% w/v methylene blue (Sigma-Aldrich, M9140) to wash. Six washed eggs were placed per well of 12-well Falcon cell culture plates containing 3 ml of dechlorinated municipal water.

Eggs were statically exposed to AhR ligands for four days with daily renewal for a final concentration of 0.5% (v/v) of vehicle (DMSO). Methylene blue was used as a fungicide in the water for the first two days of the exposures. After four days post-fertilization (dpf), larvae were maintained in clean dechlorinated municipal water with renewal every two days until either 5 or 10 dpf. The eggs were exposed to increasing nominal concentrations of BaP (5, 50, 500, 5000 ng/L), TCDD (1, 10, 100, 1000 ng/L), or vehicle control (0.5% DMSO) for the concentration-response experiment. Subsequent experiments used TCDD and BaP alone or in combination with antagonists at the following concentrations: BaP (5000 ng/L), TCDD (1 ng/L), Res (300 µg/L – upper solubility limit of Res in vehicle), or ANF (100 µg/L; Billiard et al., 2006; Timme-Laragy et al., 2007). At 5 and 10 dpf, larvae were euthanized with an overdose of MS-222 and digital photographs taken under an Olympus dissecting microscope using darkfield illumination at 40x magnification. Photographs were used to quantify fork length (distance from snout to fork of tail) at 5 dpf and to note the incidence of deformities (craniofacial abnormalities, pericardial or yolk sac edema, spinal deformities) at both 5 and 10 dpf.

4.2.3 *In vivo* Cardiac Function Determination

A total of 81 fish, divided among nine treatment groups, were used for *in vivo* analysis. Fish at 10 dpf were anesthetized by exposure to a solution of 50 mg/L MS-222 for one minute (Fritsche et al., 2000). The fish were then transferred to a small Petri dish where they were covered in a thin layer of 1% low-melt agarose (Sigma-Aldrich, A4018) containing 50 mg/L MS-222 in a lateral position. The agarose was covered with 1 ml of aerated dechlorinated municipal water at room temperature containing 50 mg/L MS-222. The Petri dish was transferred to the stage of an Olympus transilluminated upright microscope equipped with a Canon HV20 high definition digital camcorder. Larvae were imaged using brightfield illumination at 200x magnification and video footage of the beating heart was acquired for 10 minutes. Heart rate (beats per minute or bpm) was calculated by counting the number of heart beats in 10 seconds at the beginning of every minute and multiplying by six. Images were then exported from the video footage using Adobe Premiere Pro software (San Jose, CA) at 0, 2.5, 5, and 10 minutes. Ventricular cross-sectional areas at systole (sys) and diastole (dia) as well as heart length were measured using ImagePro Express software (Bethesda, MD). The percent change in cross-sectional area (ΔCSA) was calculated as a measure of cardiac function using the following equation: $\Delta CSA = ((dia-sys)/dia)*100\%$.

4.2.4 Histological Analysis

A total of n=5 larvae per treatment group were used for histological determination of heart and vessel dimensions. Fish at 10 dpf were euthanized with an overdose of MS-222 before being transferred into neutral buffered formalin. After 24 hours, fish were transferred to 70% ethanol and stored until histological analysis could be performed. Whole larvae were paraffin-embedded and sectioned (5 μm transverse sections) beginning at the anterior end of the eyes, collecting 250 serial sections, then stained with hematoxylin and eosin. Digital photomicrographs were obtained with a Zeiss AxioVert light microscope. Heart sections and sections for vascular analysis were photographed at 400x magnification. Six serial sections from the largest part of the ventricle were used to measure cardiac

dimensions and a single mean value from the sections used for each larva in statistical comparisons. Then 5 sections, each 25 μm apart, beginning near the anterior end of the stomach cavity, were used to measure the dimensions of the posterior cardinal vein and the dorsal artery and a single mean value for each vessel from the sections was used for each larva in statistical comparisons. The inner and outer diameters of the ventricular chamber, posterior cardinal vein, and dorsal artery were measured using AxioVision software. Wall thicknesses were calculated as the difference between inner and outer diameters.

4.2.5 Real-time rt-PCR

At 5 dpf, 7 fish were pooled for each real-time rt-PCR sample, while at 10 dpf, 5 fish were pooled for each sample. Real-time rt-PCR was carried out to examine expression levels of CYP1A, CYP1B1, CYP1C1, CYP1C2, COX-1, COX-2a, and COX-2b using previously described SYBR Green methods, primers, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a housekeeping gene, and the ΔΔCt method to calculate fold-expression changes compared to control (Bugiak and Weber, 2009).

4.2.6 Data and Statistics

Results are presented as mean ± standard error of the mean (SEM). Data was log-transformed (gene expression data) where necessary to meet normality and variance assumptions. Differences among treatment groups were analyzed by separate 1-way ANOVAs followed by Tukey's posteriori tests for most end-points. The groups with zero variance in the incidence data (deformities and mortalities) necessitated the use of a different posteriori test (Fisher's LSD) after 1-way ANOVA. Also, when data failed to meet normality assumptions despite a variety of transformations (fork length), a non-parametric Kruskal-Wallis test was used followed by Kolmogorov-Smirnov posteriori tests. Pearson's correlations were used to assess whether there were significant linear relationships between gene expression at 5 or 10 dpf and mortalities, deformities, cardiovascular functional end-points or histological end-points at 10 dpf. All relationships that had a $r > 0.70$ were graphed and p-values of the slope calculated. Principal

components analysis (PCA) of mean values for each treatment group was used to further assess significant relationships among all the endpoints measured. All 5 dpf gene expression values were related to all 10 dpf phenotype end-points in initial PCA analyses, and then variables with the lowest correlations were eliminated until only those with high correlation ($r > 0.65$) to components 1 and 2 remained.

4.3 Results

4.3.1 Concentration-response phenotypes

Several deformities typical of developmental AhR agonist exposure such as pericardial and yolk-sac edema, craniofacial deformities, and spinal curvature were noted to occur (Figure 4.1) in a concentration-dependent manner in developing larvae at both 5 and 10 dpf (Figure 4.2; data for 10 dpf shown). At 10 dpf, the deformity rate, but not the mortality rate remained unchanged after exposure to BaP concentrations ranging from 5 to 5000 ng/L (Figure 4.2). Higher BaP concentrations were not possible due to limited solubility. Based on these results, the concentrations for the remaining experiments were chosen to ensure adequate survival until 10 dpf (5000 ng/L BaP and 1 ng/L TCDD).

4.3.2 Mortality, Deformities, and Fork Length

After the concentration response experiments, single concentrations of AhR ligands alone and in combination were examined. The effects on mortality and deformities were similar but followed a different time-course for BaP and TCDD alone (Table 4.1). Both ANF and Res had no significant antagonistic effect on TCDD- and BaP-induced mortalities or deformities (Table 4.1). It is interesting to note that deformities were evident at 5 dpf in TCDD- and BaP/ANF-treated larvae, but that BaP or ANF treatment alone did not produce

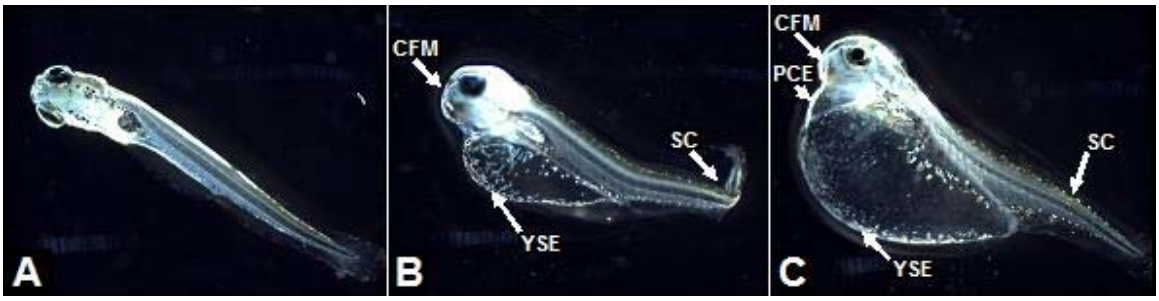


Figure 4.1: Larval zebrafish at 10 day post-fertilization (dpf) representing normal development (A) and typical deformities (B and C) associated with developmental aryl hydrocarbon receptor agonist exposure. Images are from larvae treated with vehicle (A), 1 ng/L 2,3,7,8-tetrachlorodibenzo-*p*-dioxin + 100 µg/L α-naphthoflavone (B), and 5000 ng/L benzo(a)pyrene + 100 µg/L α-naphthoflavone (C). Similar deformities to those shown here were noted at 5 dpf. All images were taken at 40x magnification. CFM – craniofacial malformation, YSE – yolk sac edema, SC – spinal curvature, PCE – pericardial edema

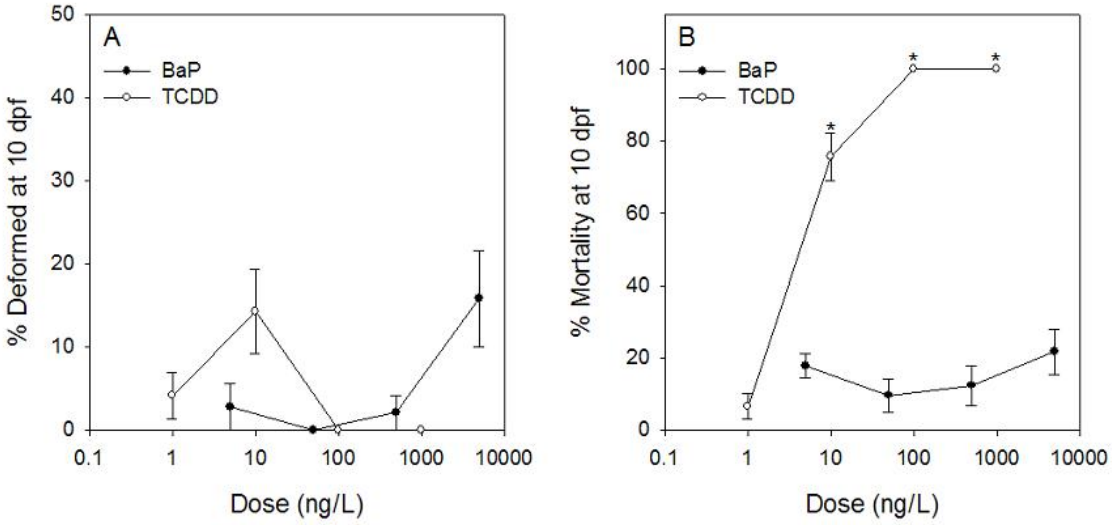


Figure 4.2: Deformity (A) and mortality (B) rates at 10 days post-fertilization (dpf) after exposure to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; black points) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; white points) at increasing concentrations. N=12 wells per treatment group, each with 6 larvae/well. Results are mean ± SEM. Asterisks indicates significant differences from vehicle control deformity incidence of 3.1 ± 2.1% or mortality incidence of 15.8 ± 3.5% at 10 dpf using Tukey's posteriori test after 1-way ANOVA.

visibly detectable deformities until 10 dpf (Table 4.1). Furthermore, BaP, TCDD, and Res had no significant effect on fork length at 5 dpf (Figure 4.3). However, ANF alone or in combination with BaP significantly decreased fork length when compared to the control group (Figure 4.3).

4.3.3 Functional and Histological Analysis of the Heart

Using digital image analyses from videomicroscopy of live larvae at 10 dpf, AhR ligand exposure showed no significant effects on heart rate, ventricular length or heart function (indicated by percent change in ventricular cross-sectional area or ΔCSA) (Table 4.2). However, histological analysis of the heart at 10 dpf (Table 4.2; Figure 4.4) indicated that BaP in combination with ANF significantly reduced the ventricular chamber width, but not ventricular wall thickness. All other treatments were not significantly different compared to the control group for all cardiac end-points (Table 4.2).

4.3.4 Histological Analysis of Dorsal Aorta and Posterior Cardinal Vein Dimensions

While ANF exposure had no antagonistic effects against TCDD, when combined with BaP, ANF significantly increased luminal diameter of both dorsal aorta and posterior cardinal vein compared to that of the control group (Table 4.3; Figure 4.4). No other treatment had a significant effect on blood vessel luminal diameter compared to control (Table 4.3). In contrast, no significant differences in wall thickness of both the dorsal aorta (DA) and posterior cardinal vein (PCV) were detected among all treatment groups in 10 dpf zebrafish (Table 4.3; Figure 4.4).

4.3.5 Effects of AhR Ligands on mRNA Expression

At 5 dpf, TCDD, BaP, and ANF exposure significantly increased expression of CYP1A in whole larvae, while TCDD decreased CYP1C2 mRNA relative to control groups (Figure 4.5). Resveratrol alone did not have a significant effect on mRNA expression of

Table 4.1: Mortality and deformity rates of developing zebrafish at 5 and 10 days post-fertilization (dpf) after exposure to the aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with resveratrol (Res; 300 μg/L) or α-naphthoflavone (ANF; 100 μg/L). Data is expressed as mean ± SEM. n = 12 wells per group, each with 6 larvae per well. **$p<0.01$ in Fisher's LSD posteriori test after 1-way ANOVA. Please note that deformity data at 10 dpf is not cumulative deformities and is the deformity rate in the surviving larvae at 10 dpf. Presumably, severely deformed larvae have died prior to this time point, although this was not quantified. A separate cohort with its own frequencies of mortality and deformities (also not cumulative) at 5 dpf is reported.

Treatment	5 dpf		10 dpf	
	% Deformities	% Mortality	% Deformities	% Mortality
Control	3.3 ± 2.3	3.5 ± 2.4	3.3 ± 2.3	1.4 ± 1.4
Res	8.2 ± 2.6	2.1 ± 2.1	1.7 ± 1.7	5.0 ± 2.6
ANF	14.9 ± 6.6	3.5 ± 2.4	5.0 ± 3.6	7.5 ± 4.5
BaP	0.0 ± 0.0	0.0 ± 0.0	15.8 ± 5.8**	20.6 ± 5.8**
BaP & Res	7.2 ± 3.1	4.4 ± 2.3	0.0 ± 0.0	2.8 ± 1.9
BaP & ANF	31.0 ± 8.2**	5.8 ± 3.2	0.0 ± 0.0	21.4 ± 6.6**
TCDD	26.1 ± 8.7**	0.0 ± 0.0	1.4 ± 1.4	6.5 ± 3.5
TCDD & Res	15.1 ± 5.8	1.4 ± 1.4	2.8 ± 2.8	11.4 ± 4.5
TCDD & ANF	22.5 ± 6.9**	1.4 ± 1.4	4.4 ± 3.1	10.0 ± 3.7

Table 4.2: Functional and morphological analyses of hearts in 10 days-post-fertilization (dpf) zebrafish determined using either *in vivo* microscopy or histological techniques. Larvae were exposed to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with resveratrol (Res; 300 μg/L) or α-naphthoflavone (ANF; 100 μg/L). ΔCSA = percent change in ventricular cross-sectional area from diastole to systole. Data are mean ± SEM. **p<0.01 compared to control in Tukey's posteriori test after 1-way ANOVA. n=8-10 per group.

	In vivo Microscopy					Histology	
Treatment	Heart Rate (beats/min)	Ventricular Systolic Area (μm^2)	Ventricular Diastolic Area (μm^2)	Ventricular ΔCSA (%)	Ventricular Length (μm)	Ventricular Wall Thickness (μm)	Ventricular Inner Diameter (μm)
Control	134 ± 4	4970 ± 545	8451 ± 1611	36.3 ± 5.3	120 ± 6	7.18 ± 0.21	46.5 ± 2.2
Res	129 ± 3	5105 ± 398	6949 ± 637	25.4 ± 3.4	115 ± 6	6.80 ± 0.36	39.8 ± 2.9
ANF	139 ± 10	3267 ± 297	6012 ± 745	42.8 ± 4.3	97 ± 8	7.95 ± 0.33	42.6 ± 3.8
BaP	135 ± 8	4882 ± 664	7284 ± 731	34.4 ± 3.0	113 ± 5	8.38 ± 0.41	42.5 ± 2.7
BaP & Res	136 ± 5	4690 ± 320	7826 ± 448	38.9 ± 4.6	112 ± 4	6.78 ± 0.56	41.2 ± 2.1
BaP & ANF	131 ± 13	4521 ± 786	7995 ± 1536	37.9 ± 6.0	103 ± 12	8.94 ± 0.34	24.9 ± 8.2**
TCDD	143 ± 4	4415 ± 306	6635 ± 241	33.5 ± 3.5	101 ± 3	8.32 ± 0.18	39.7 ± 2.8
TCDD & Res	154 ± 3	4294 ± 224	7028 ± 634	34.6 ± 3.8	104 ± 5	8.40 ± 0.22	37.2 ± 3.4
TCDD & ANF	140 ± 6	3523 ± 230	5552 ± 317	35.8 ± 4.7	97 ± 5	8.53 ± 0.44	31.8 ± 5.1

Table 4.3: Histological analysis of dorsal aorta and posterior cardinal vein in 10 days post-fertilization (dpf) zebrafish. Vessel wall thickness and luminal diameters after larvae were exposed to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with antagonists resveratrol (Res; 300 μg/L) and α-naphthoflavone (ANF; 100 μg/L). Data are mean ± SEM. **p<0.01 compared to control in Tukey's posteriori test after 1-way ANOVA. n=5 per group

Treatment	Dorsal Aorta		Posterior Cardinal Vein	
	Thickness (μm)	Luminal Diameter (μm)	Thickness (μm)	Luminal Diameter (μm)
Control	1.78 ± 0.02	7.34 ± 0.58	1.57 ± 0.08	7.05 ± 1.42
Res	1.93 ± 0.13	7.86 ± 0.64	1.74 ± 0.06	6.57 ± 0.40
ANF	1.80 ± 0.12	10.37 ± 1.05	1.49 ± 0.17	10.00 ± 0.88
BaP	1.50 ± 0.10	9.60 ± 0.39	1.27 ± 0.08	9.81 ± 0.83
BaP & Res	1.58 ± 0.06	6.57 ± 0.60	1.48 ± 0.09	8.91 ± 1.49
BaP & ANF	1.77 ± 0.24	14.04 ± 2.99**	1.62 ± 0.15	13.52 ± 2.44**
TCDD	1.61 ± 0.23	7.88 ± 0.81	1.34 ± 0.07	7.36 ± 0.78
TCDD & Res	1.53 ± 0.05	8.16 ± 0.83	1.21 ± 0.09	5.36 ± 0.42
TCDD & ANF	1.55 ± 0.07	8.34 ± 0.98	1.39 ± 0.16	9.40 ± 1.20

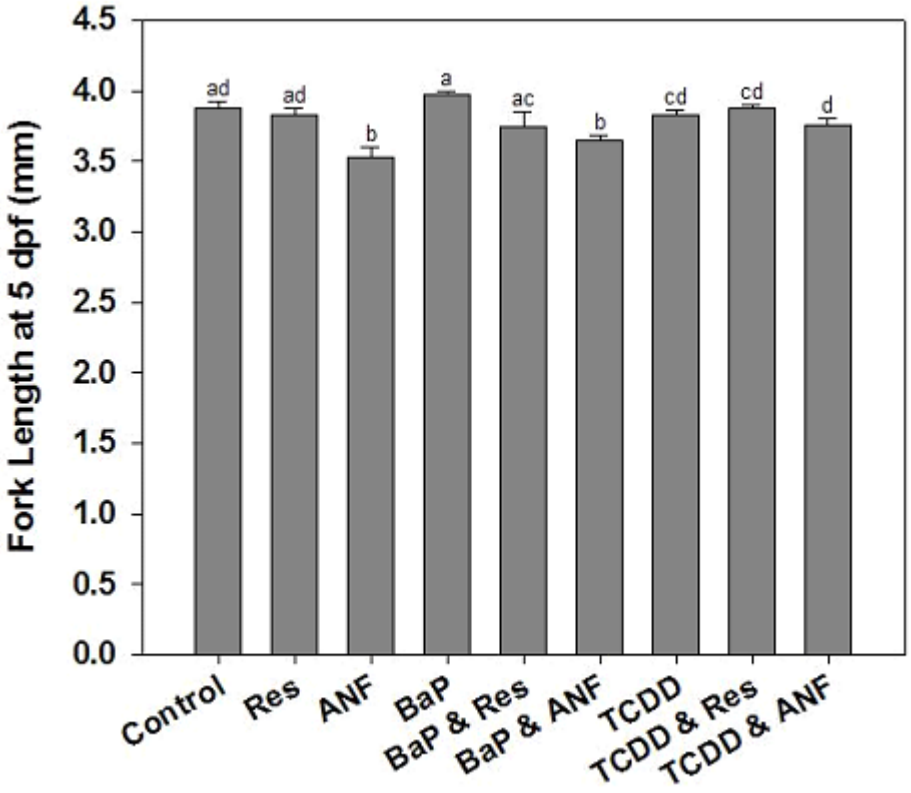


Figure 4.3: Fork length determined in zebrafish larvae at 5 days post-fertilization (dpf) from digital image analyses taken at 40x magnification. Data are mean ± SEM. Larvae were exposed to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with resveratrol (Res; 300 µg/L) or α-naphthoflavone (ANF; 100 µg/L). Different letters indicate statistically significant ($p < 0.05$) differences between treatment groups in Kolmogorov-Smirnov test after Kruskal Wallis non-parametric analysis. n=29-49 larvae/treatment group.

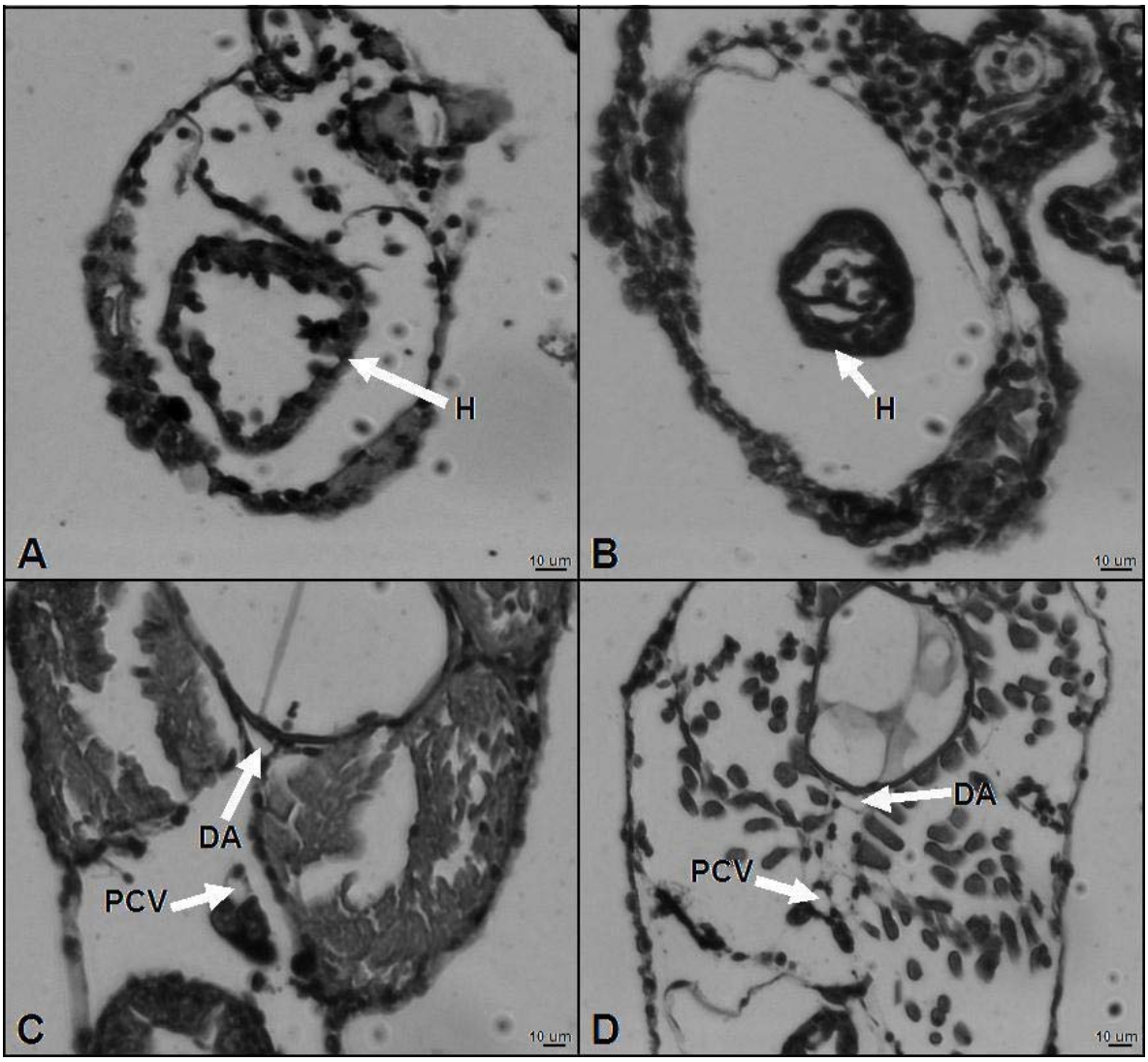


Figure 4.4: Representative histological cross sections stained with hematoxylin and eosin of whole zebrafish larvae showing the heart (A,B) and major blood vessels (C,D). Sections are from larvae exposed developmentally to vehicle (A, C), 5000 ng/L benzo(a)pyrene + 100 µg/L α-naphthoflavone (B), and 1 ng/L 2,3,7,8-tetrachlorodibenzo-*p*-dioxin + 100 µg/L α-naphthoflavone (D). H – heart, DA – dorsal aorta, PCV – posterior cardinal vein.

any gene examined nor did it have any effect on BaP- or TCDD-induced mRNA expression changes. In contrast, ANF caused an apparently additive increase in CYP1A expression with both BaP and TCDD, as well as an additive increase in CYP1C1 with TCDD rather than exerting any antagonistic effect at 5 dpf (Figure 4.5). At 10 dpf, CYP1A expression remained significantly elevated compared to control in both BaP- and TCDD-treated groups (Figure 4.6). However, no other genes examined remained significantly altered by these ligands compared to control groups at 10 dpf (Figure 4.6).

4.3.6 Correlation and Principal Component Analyses

As an initial step in principal components analyses, all end-points measured in the previous figures were regressed against each other using mean values from each treatment group (± SEM) to uncover relationships among the end-points. Of particular interest was how mRNA expression of each gene analyzed at 5 dpf correlated with mortality and deformity rates at both 5 and 10 dpf (Table 4.4), or how 10 dpf gene expression was correlated with mortality and deformity rates at that same time. All linear relationships with a Pearson correlation coefficient $r \geq 0.7$ are shown (Figure 4.7). At 5 dpf, CYP1C2 expression was negatively and COX-1 expression was positively correlated to the deformity rate at 5 dpf (Figure 4.7). Furthermore, COX-2b expression at 5 dpf was highly negatively correlated with the mortality rate at 10 dpf (Figure 4.7). The BaP/ANF co-exposure group is indicated with an arrow in these figures because it was consistently a group that was on the extreme end of each relationship (Figure 4.7).

Gene expression at 10 dpf failed to show significant relationships to the functional and histological end-points measured at the same time point (Table 4.5). In contrast, 5 dpf gene expression showed several significant relationships to the 10 dpf phenotype (Figure 4.8). Specifically, ventricular chamber width at 10 dpf was negatively correlated with CYP1A expression at 5 dpf, but positively correlated with CYP1C2 and COX-2a expression at 5 dpf. The CYP1C1 expression at 5 dpf was negatively correlated with

Figure 4.5: CYP1A, CYP1B1, CYP1C1, CYP1C2, COX-1, COX-2a, and COX-2b mRNA expression (fold change relative to control levels) in pooled whole zebrafish larvae at 5 days post-fertilization (dpf). Data are mean ± SEM. Larvae were exposed to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with resveratrol (Res; 300 µg/L) or α-naphthoflavone (ANF; 100 µg/L). Different letters indicate statistically significant ($p < 0.05$) differences between treatment groups in Tukey's posteriori test after 1-way ANOVA. In panels with no letters, no significant differences were detected among treatments. n=4-6 samples/treatment group.

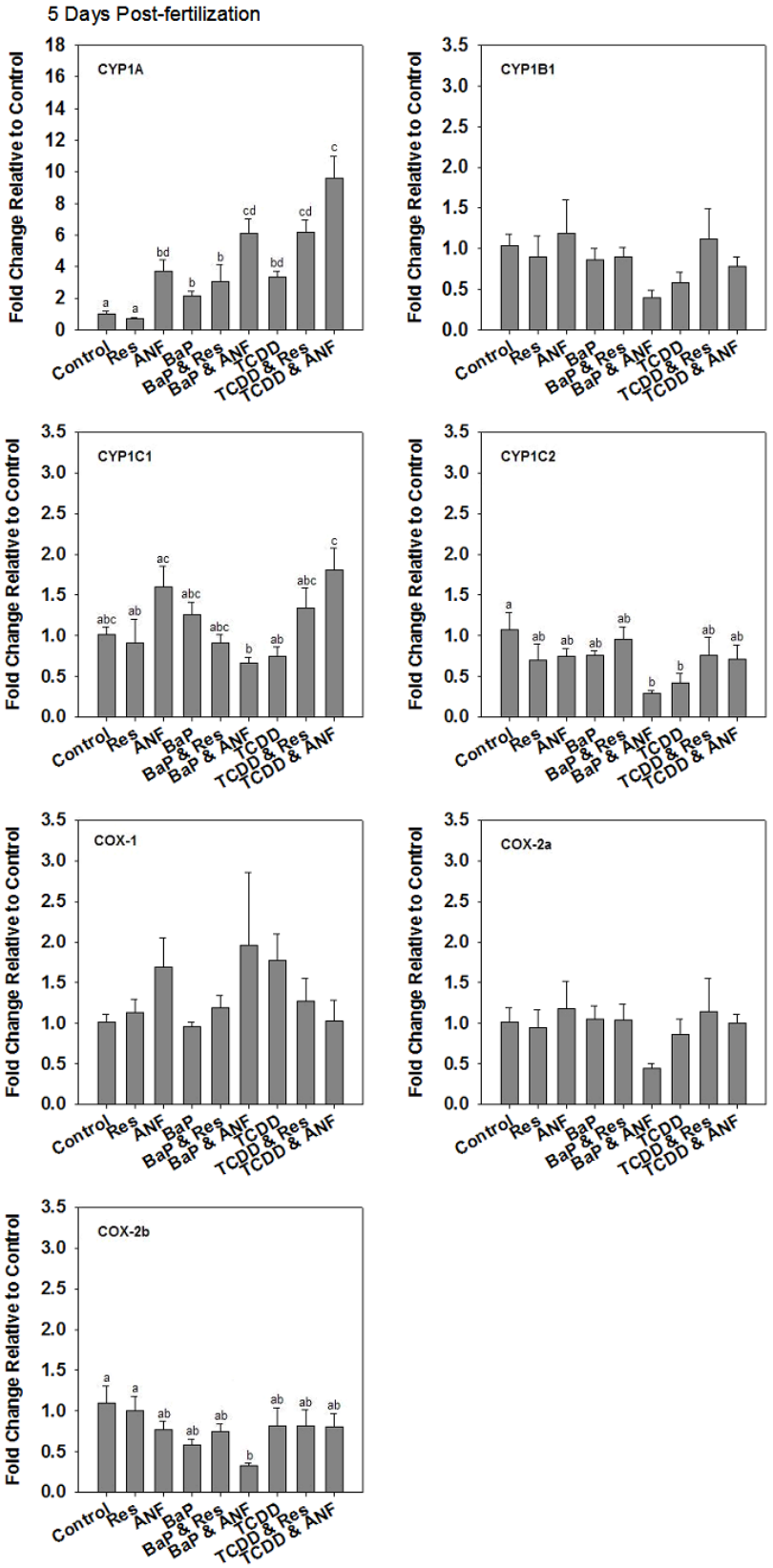
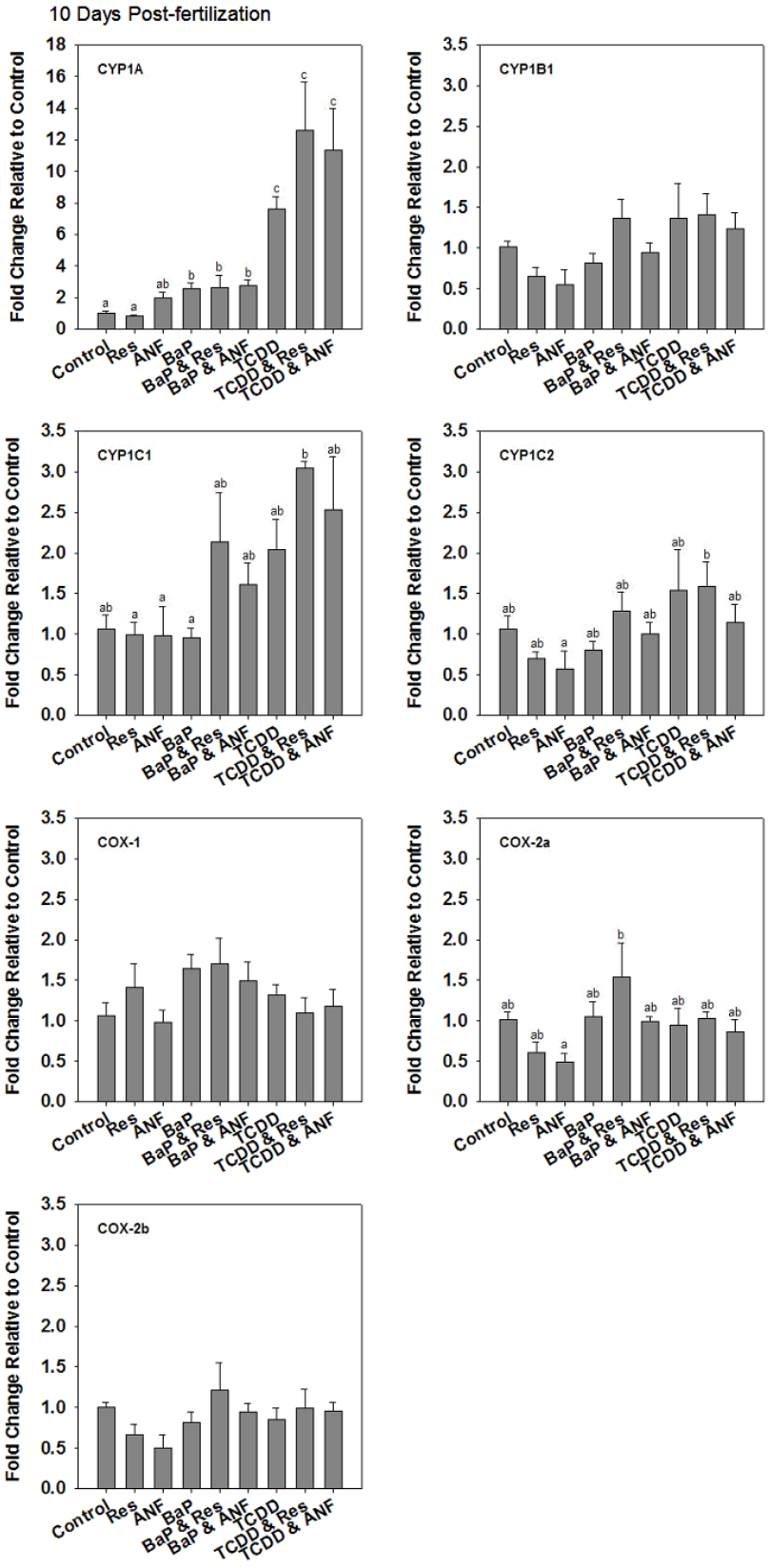

5 Days Post-fertilization
CYP1A
CYP1B1
CYP1C1
CYP1C2
COX-1
COX-2a
COX-2b
Fold Change Relative to Control
Control
Res
ANF
BaP
BaP & Res
BaP & ANF
TCDD
TCDD & Res
TCDD & ANF

Figure 4.6: CYP1A, CYP1B1, CYP1C1, CYP1C2, COX-1, COX-2a, and COX-2b mRNA expression (fold change relative to control levels) in pooled whole zebrafish larvae at 10 days post-fertilization (dpf). Data are mean ± SEM. Larvae were exposed from fertilization until 4 dpf to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with resveratrol (Res; 300 μg/L) or α-naphthoflavone (ANF; 100 μg/L). Different letters indicate statistically significant ($p < 0.05$) differences between treatment groups in Tukey's posteriori test after 1-way ANOVA. In panels with no letters, no significant differences were detected among treatments. n=4-6 samples/treatment group.


10 Days Post-fertilization
CYP1A
CYP1B1
CYP1C1
CYP1C2
COX-1
COX-2a
COX-2b
Fold Change Relative to Control
Control
Res
ANF
BaP
BaP & Res
BaP & ANF
TCDD
TCDD & Res
TCDD & ANF

ventricular cross-sectional area at systole and diastole at 10 dpf, while CYP1A expression at 5 dpf was negatively correlated with ventricular length at 10 dpf. Overall, the BaP/ANF co-exposure group was observed to drive many of these linear relationships (BaP/ANF group indicated by arrows in Figure 4.8).

The gene expression levels of each gene at 5 and 10 dpf were correlated with histological measurements of the dorsal aorta and posterior cardinal vein at 10 dpf (Table 4.6). COX-2b mRNA expression at 5 dpf was strongly negatively correlated with luminal diameters of both dorsal aorta and posterior cardinal vein in developing zebrafish at 10 dpf (Figure 4.9). Also, CYP1A expression at 10 dpf was negatively correlated with posterior cardinal vein wall thickness (Figure 4.9). Again, the BaP/ANF co-exposure group appears to drive several of the linear relationships (Figure 4.9).

From the preceding correlation analyses, gene expression at 5 dpf correlated to many different aspects of phenotype at 10 dpf, while 10 dpf gene expression showed only one correlation to phenotype. Therefore, principal components analysis was performed on functional and morphological data at 10 dpf along with mRNA expression data for only 5 dpf. Principal components analysis provides a further examination of the relationships among multiple end-points, rather than being limited to just two end-points at a time as occurs with linear regressions. The top 12 end-points that explained the greatest variance among the groups (74% of the variability explained by components 1 and 2) were included in the final analysis (Table 4.7). The component loadings shown in Table 4.7 indicate the *r*-value or strength of the end-point's correlation to each component. Furthermore, the relationships of the end-points to each other can be inferred through their respective relationships to each component. In order to keep CYP1A in the final principal components analysis, the cut-off for inclusion of an end-point was relaxed to $r = 0.65$. The first component contained a cluster of end-points that all corresponded to ventricular morphology and blood vessel luminal diameter, while the second component contained only blood vessel wall thickness and heart rate for morphological and functional end-points. Ventricular wall thickness and luminal diameter of both blood vessels as well as CYP1A expression were positively correlated to component 1 (Table 4.7). In contrast,

Table 4.4: Correlations between mRNA expression and mortality and deformity rates in zebrafish larvae at 5 and 10 dpf (Pearson correlation coefficient *r*).

5 days post fertilization			10 days post fertilization			10 days post fertilization		
Genes	Mortality	Deformities	Mortality	Deformities		Genes	Mortality	Deformities
CYP1A	0.02	0.67	0.36	-0.14		CYP1A	0.11	-0.09
CYP1B1	-0.12	-0.64	-0.47	0.23		CYP1B1	-0.16	-0.35
CYP1C1	-0.31	-0.12	0.01	0.42		CYP1C1	-0.01	-0.37
CYP1C2	-0.001	-0.83	-0.59	0.20		CYP1C2	-0.14	-0.38
COX-1	0.37	0.77	0.25	-0.46		COX-1	0.33	0.14
COX-2a	-0.44	-0.63	-0.47	0.37		COX-2a	-0.02	-0.10
COX-2b	-0.29	-0.45	-0.85	-0.15		COX-2b	-0.10	-0.29

Table 4.5: Correlations between mRNA expression at 5 or 10 dpf and cardiac dimensions at 10 dpf measured using *in vivo* microscopy and histological techniques (Pearson correlation coefficient *r*).

5 dpf	Measurements at 10 dpf					
Genes	Wall Thickness	Inner Diameter	Length	Systolic Area	Diastolic Area	Ejection Fraction
CYP1A	0.68	-0.73	-0.78	-0.67	-0.51	0.31
CYP1B1	-0.44	0.69	0.17	-0.2	-0.15	0.15
CYP1C1	0.22	0.08	-0.46	-0.72	-0.71	0.29
CYP1C2	-0.67	0.74	0.55	0.16	0.22	0.1
COX-1	0.44	-0.47	-0.56	-0.31	-0.04	0.35
COX-2a	-0.37	0.73	0.06	-0.25	-0.36	0.09
COX-2b	-0.66	0.67	0.38	0.14	-0.08	-0.37
10 dpf	Measurements at 10 dpf					
Genes	Wall Thickness	Inner Diameter	Length	Systolic Area	Diastolic Area	Ejection Fraction
CYP1A	0.53	-0.36	-0.58	-0.45	-0.51	-0.02
CYP1B1	0.14	-0.16	-0.14	0.02	0.07	0.04
CYP1C1	0.32	-0.41	-0.46	-0.29	-0.24	0.06
CYP1C2	0.2	-0.15	-0.15	0.06	0.1	-0.03
COX-1	-0.13	-0.16	0.35	0.55	0.37	-0.2
COX-2a	-0.15	0.01	0.33	0.4	0.56	0.18
COX-2b	-0.1	-0.16	0.26	0.32	0.5	0.11

Table 4.6: Correlations between mRNA expression and dorsal aorta (DA) and posterior cardinal vein (PCV) dimensions as ascertained through histological and functional analysis at 10 dpf (Pearson correlation coefficient *r*)

5 dpf	Measurements at 10 dpf			
Genes	Dorsal Aorta		Posterior Cardinal Vein	
	Thickness	Inner Diameter	Thickness	Inner Diameter
CYP1A	-0.45	0.3	-0.38	0.31
CYP1B1	0.04	-0.49	-0.16	-0.56
CYP1C1	-0.32	-0.12	-0.41	-0.08
CYP1C2	-0.08	-0.69	-0.02	-0.48
COX-1	0.27	0.64	0.14	0.47
COX-2a	-0.27	-0.67	-0.42	-0.62
COX-2b	0.26	-0.8	0.15	-0.83
10 dpf	Measurements at 10 dpf			
Genes	Dorsal Aorta		Posterior Cardinal Vein	
	Thickness	Inner Diameter	Thickness	Inner Diameter
CYP1A	-0.64	-0.17	-0.7	-0.31
CYP1B1	-0.67	-0.42	-0.54	-0.31
CYP1C1	-0.61	-0.22	-0.56	-0.26
CYP1C2	-0.59	-0.36	-0.56	-0.39
COX-1	-0.23	0.06	0.07	0.35
COX-2a	-0.58	-0.29	-0.29	0.02
COX-2b	-0.5	-0.31	-0.21	-0.06

Table 4.7: Principal components analysis of functional and morphological data from 10 dpf along with mRNA expression at 5 dpf in zebrafish exposed developmentally to aryl hydrocarbon receptor ligands. The top 12 variables explaining the greatest variance were included in the final analysis and are listed in descending order along with correspondent component loadings. Component loadings represent the strength of correlation of that end-point to each component. Furthermore, the relationships of the variables to each other can be inferred through their respective relationships to each component. DA – dorsal aorta; PCV – posterior cardinal vein.

	Component 1	***Component 2***
Total Variance Explained	44%	30%
Variable	**Component Loadings**	
Ventricular Thickness	0.903	0.204
Ventricular Inner Diameter	-0.837	0.252
COX-2b mRNA at 5 dpf	-0.827	0.280
CYP1A mRNA at 5 dpf	0.771	0.390
DA Inner Diameter	0.765	-0.515
CYP1C2 mRNA at 5 dpf	-0.764	0.328
Ventricular Length	-0.758	-0.314
PCV Inner Diameter	0.680	-0.534
Heart Rate	0.185	0.872
PCV Thickness	-0.282	-0.863
DA Thickness	-0.311	-0.751
CYP1C1 mRNA at 5 dpf	0.141	0.624

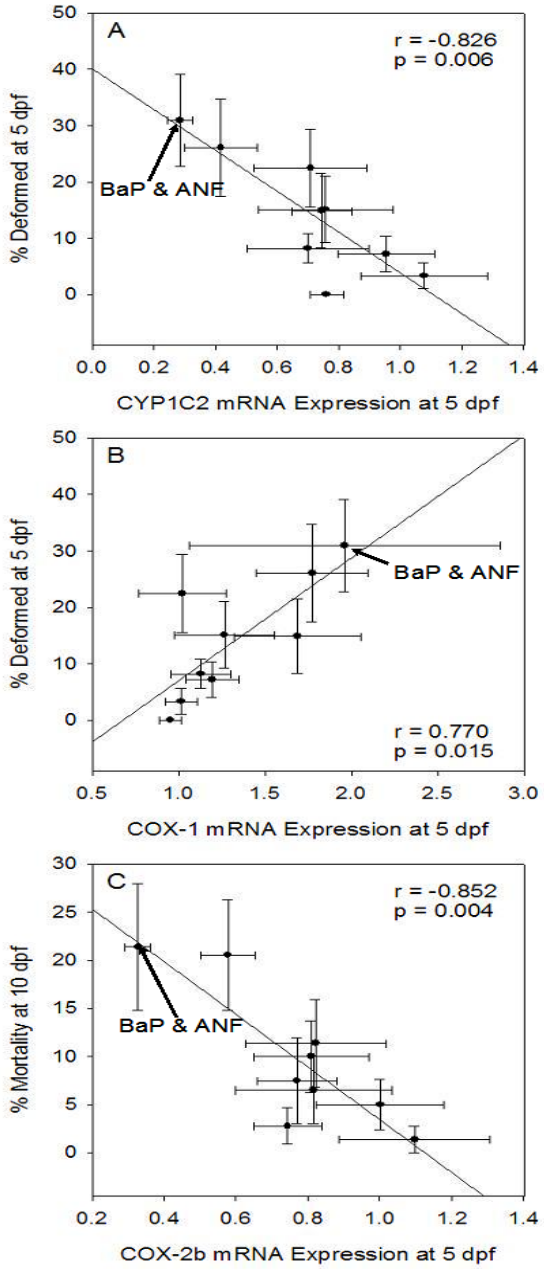


Figure 4.7: Correlations with Pearson correlation coefficient $r \geq 0.7$ are shown. Relationships between (A) CYP1C2 and (B) COX-1 mRNA expression at 5 days post-fertilization (dpf) with deformity rates at 5 dpf or (C) COX-2b mRNA expression at 5 dpf with mortality at 10 dpf. Larvae were exposed to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with resveratrol (Res; 300 µg/L) or α-naphthoflavone (ANF; 100 µg/L). Data are mean ± SEM. Pearson correlation coefficients (*r*) and associated p values are indicated in the respective panels.

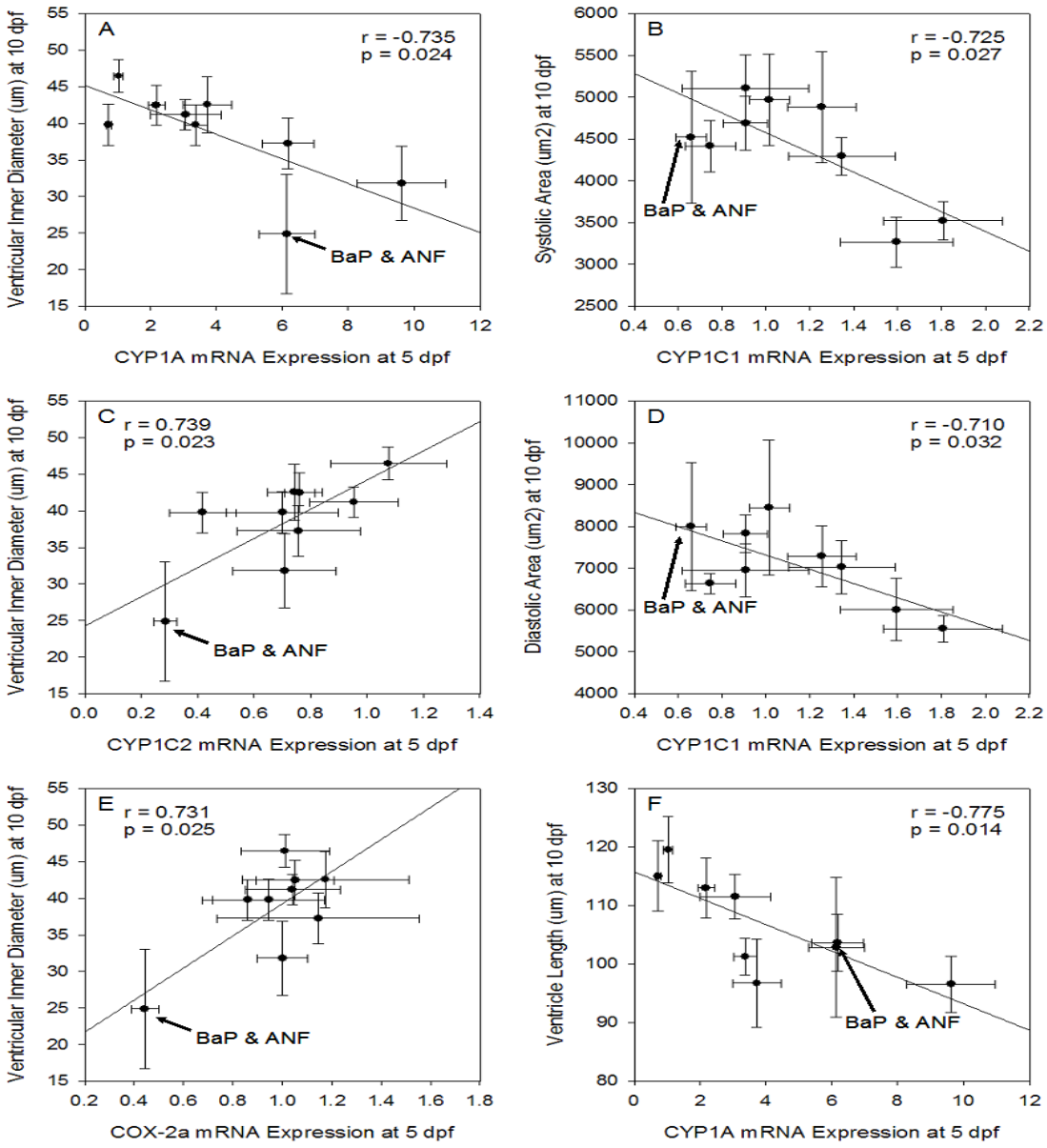


Figure 4.8: Correlations (Pearson correlation coefficient $r \geq 0.7$) between (A) CYP1A, (C) CYP1C2, and (E) COX-2a mRNA expression at 5 days post-fertilization (dpf) with ventricular chamber width at 10 dpf, (B) CYP1C1 mRNA expression at 5 dpf with 2-dimensional systolic area, (D) CYP1C1 mRNA expression at 5 dpf with 2-dimensional diastolic area, and (F) CYP1A mRNA expression at 5 dpf with heart length at 10 dpf after 96 hour exposure to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with resveratrol (Res; 300 µg/L) or α-naphthoflavone (ANF; 100 µg/L). Data are mean ± SEM. Pearson correlation coefficients (*r*) and associated p values are indicated in the respective panels. Arrows indicate BaP and ANF treatment group value.

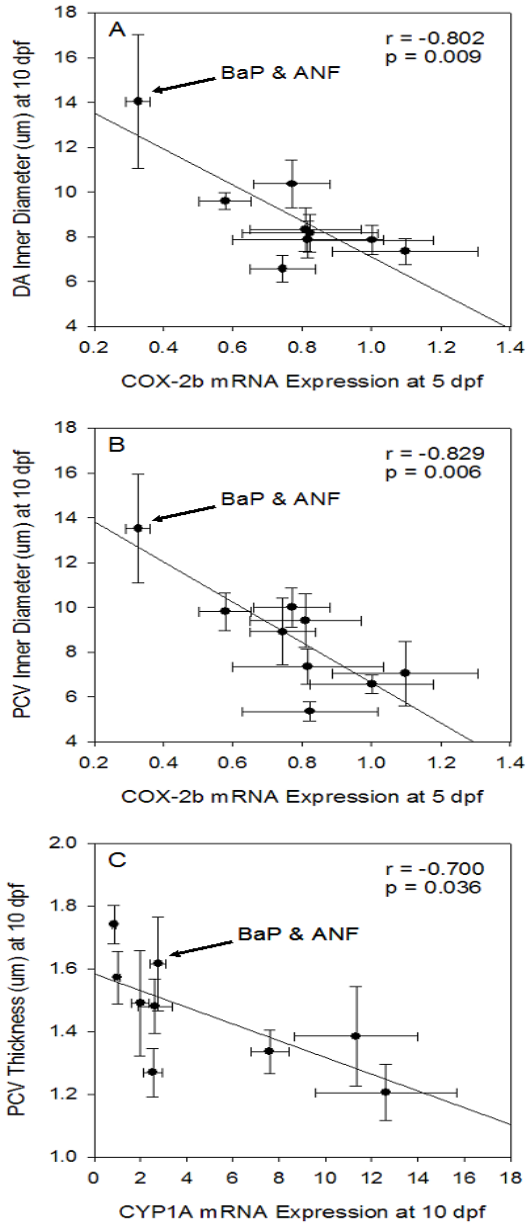


Figure 4.9: Correlations ($r \geq 0.7$) between (A) COX-2b mRNA expression at 5 days post-fertilization (dpf) with dorsal aorta (DA) inner diameter at 10 dpf, (B) COX-2b mRNA expression at 5 dpf with posterior cardinal vein (PCV) inner diameter at 10 dpf, and (C) CYP1A mRNA expression at 10 dpf with posterior cardinal vein wall thickness at 10 dpf after 96 hour exposure to aryl hydrocarbon receptor agonists benzo(a)pyrene (BaP; 5000 ng/L) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 1 ng/L) alone and in combination with antagonists resveratrol (Res; 300 µg/L) or α-naphthoflavone (ANF; 100 µg/L). Data are mean ± SEM. Pearson correlation coefficients (*r*) and associated p values are indicated in the respective panels. Arrows indicate BaP and ANF treatment group value.

ventricular inner diameter and ventricular length, along with CYP1C2 and COX-2b expression were negatively correlated to component 1 (Table 4.7). For component 2, heart rate and CYP1C1 were positively correlated, while wall thickness of both blood vessels was inversely related to this component.

4.4 Discussion

The first important finding of this study is that gene expression at 5 dpf correlates with morphological and functional phenotypes observed at 10 dpf in larval zebrafish exposed developmentally to AhR ligands. However, the most striking finding of this study is that expression of CYP1A is positively linked, while COX2b and CYP1C2 is negatively related to a deformed cardiac phenotype and increased blood vessel luminal diameter. In contrast, only one gene examined, CYP1C1, is negatively associated with blood vessel wall thickness, but is positively linked to heart rate. While there have been numerous studies examining the effects of AhR agonists on larval gene expression and developmental deformities associated with AhR agonist exposure, few have attempted phenotypic anchoring by relating these two types of endpoints. Finally, this study has shown that BaP and TCDD mediate similar changes, with differing efficacies, but that BaP co-treatment with ANF seems to produce an additive or possibly unique interaction that is worthy of further investigation.

While there have been many studies involving zebrafish and AhR-mediated developmental toxicity, most have used high doses of micro-injected AhR agonist, higher aqueous exposures or examined earlier time-points than the current study (Belair et al., 2001; Andreasen et al., 2002a; Teraoka et al., 2002; Carney et al., 2004, 2006; Dong et al., 2004; Antkiewicz et al., 2005; Mehta et al., 2008). One of the main goals of this study was to assess morphological and functional end-points at a later point (10 dpf) than most studies. A recent study reported a threshold for prolonged zebrafish survival at 50 pg/ml TCDD aqueous exposure (King Heiden et al., 2009), which is close to the 1 ng/L (=1 pg/ml) chosen based on the concentration-response experiment in the current study. The

previous study reported slightly higher incidences of deformities at comparable aqueous exposures than the current study (King Heiden et al., 2009). This difference in deformity rates may be due to the more detailed digital quantitation of craniofacial deformities and edema in the previous study compared to the current study. However, since the mortality rates are also much lower in the current study, the possibility that the actual aqueous TCDD concentrations achieved in the current study were lower than the nominal concentration cannot be excluded. Despite these differences in apparent TCDD sensitivity, the current study agrees well with this previous study on many points including the finding that the majority of mortalities occurred between 5 and 10 dpf. Furthermore, ANF exposure, alone or in combination with AhR agonists, caused significantly decreased fork length, agreeing with previous studies that also reported larval growth retardation after AhR agonist exposure (Prasch et al., 2003; King Heiden et al., 2009).

One of the most common cardiac effects reported in fish after developmental AhR agonist exposure is decreased heart rate or cardiac output (Henry et al., 1997; Andreasen et al., 2002a; Antkiewicz et al., 2005; Carney et al., 2006; Yamauchi et al., 2006). However, the current study found no significant effect on heart rate or heart function. The reasons for this lack of observed change in heart function are not clear, but agree with the lower deformity and mortality rate also observed in the current study. The longer ventricle with thicker walls and thinner diameter observed in the current study after developmental co-exposure to BaP/ANF is consistent with the unlooped heart phenotype reported in a previous TCDD study (Antkiewicz et al., 2005). However, another study that used several PAHs instead reported dilated ventricular chambers and thinner walls in larval zebrafish (Incardona et al., 2004). The type of PAH used and the presence of additional AhR ligands are likely to be responsible for the observed differences in heart phenotype. The potent combination of BaP/ANF co-exposure produced a tube-like ventricular phenotype alongside impaired somatic growth (from Figure 4.4, panel D, skeletal muscle cells appear hypoplastic), suggesting these two processes may be related. In contrast, Res failed to exert any effect in the current study, neither antagonist or agonistic, agreeing with a previous study in adult zebrafish from this laboratory (Bugiak and Weber, 2009). Furthermore, the synergistic action of BaP/ANF co-exposure is similar to previous studies using other PAHs,

such as β-naphthoflavone, combined with ANF (Wassenberg and Di Giulio, 2004; Billiard et al., 2006; Timme-Laragy et al., 2007). Since the BaP/ANF group stood out in most correlations examined in the current study, it supports previous suggestions that there is synergistic activation of the same pro-oxidant mechanism (Timme-Laragy et al., 2009). While these previous studies have reported synergistic mRNA increases when ANF is combined with a PAH, CYP1A enzyme activity may actually be inhibited in these co-exposures (Wassenberg and Di Giulio, 2004; Timme-Laragy et al., 2007). Thus, the combined exposure of these agents may be producing a unique mechanism of toxicity or may be simulating the mechanism of TCDD toxicity.

Peripheral vascular bed health and function have been reported to decline after developmental exposure to AhR agonists possibly secondary to decreased cardiac output (Henry et al., 1997; Incardona et al., 2004) while embryonic vascular structures fail to properly regress (Hornung et al. 1999; Bello et al., 2004; Goldstone and Stegeman, 2006). In the current study, increased luminal diameter of both dorsal aorta and posterior cardinal vein was observed only after co-exposure to BaP and ANF. The failure to see similar blood vessel changes in TCDD and BaP-treated groups could again be due to the lower concentrations used in the current study or due to the fact that only large vessels were examined compared to much smaller vessels in previous studies. The longer heart of smaller diameter observed in the current study would cause a decrease in cardiac output and should lead to thinner blood vessel walls since pressure is a major stimulus for increasing wall thickness. Instead, the principal components analysis suggests that heart rate, not the unlooped heart phenotype, is inversely related to vessel wall thickness. Previous reports of AhR-mediated developmental deformities have instead indicated a reduction in blood flow occurs that eventually leads to vascular stasis (Guiney et al., 1997; Henry et al., 1997; Belair et al., 2001; Dong et al., 2002, 2004). The larger luminal diameter of the large conduit vessels observed in the current study would be consistent with this since dilation would slow blood flow velocity, particularly to peripheral vascular beds.

Dioxin and, to a lesser extent, BaP are both known agonists of the AhR (Ortiz-Delgado and Sarasquete, 2004) and both induce increased expression of CYP1A (Guiney et al., 2000; Andreasen et al., 2002a; Teraoka et al., 2003; Billiard et al., 2006; Wang et al.,

2006; Jönsson et al., 2007a, b; Bugiak and Weber, 2009), agreeing with the results of our current study. In larvae, CYP1A expression has been reported to peak at 3 dpf after TCDD exposure and then decline (Prasch et al., 2003). The persistence of TCDD- and BaP-induced CYP1A mRNA levels from 5 until 10 dpf in the current study could be explained by these agonists persisting in a reservoir within exposed tissues or another unknown reservoir within the exposure system (Belair et al., 2001).

Based on the results of the current study, CYP1C2 and COX-2b may have a protective effect against the CYP1A- and AhR-mediated developmental ventricular deformities and mortalities. Furthermore, CYP1C1 may be protective against AhR-mediated decreases in heart rate and increases in large blood vessel diameter. Although COX-1 expression was not strongly related to either component in principal component analyses, it appears detrimental based on regression analyses in the current study. Previous studies have shown that there are multiple genes responsive to AhR stimulation (Carney et al., 2006; Bugiak and Weber, 2009; Teraoka et al., 2009), but the normal physiological roles of many of these genes are still uncertain. CYP1C1 and CYP1C2, for example, are still relatively new genes exclusive to fish and their roles in development are unknown (Godard et al., 2005; Wang et al., 2006; Jönsson et al., 2007a, b), but have been suggested previously to contribute to AhR-mediated developmental toxicity (Antkiewicz et al., 2006). In contrast, CYP1B1 is generally associated with smooth muscle (Kerzee and Ramos, 2001), but its expression has been shown to increase in liver and vascular tissue after adult exposure to AhR agonists (Bugiak and Weber, 2009). However, there was no indication in the current study or other studies (Timme-Laragy et al., 2008; Yin et al., 2008) that CYP1B1 is required for AhR-mediated cardiovascular deformities.

Constitutive COX-1, widely expressed throughout developing zebrafish larvae (Pini et al., 2005), has been implicated in normal cardiac development (Cha et al., 2005, 2006) and has been shown to increase in response to AhR agonist exposure in adult zebrafish tissue (Bugiak and Weber, 2009). However, the results of the current study are equivocal regarding a role for COX-1 in AhR-mediated developmental abnormalities since COX-1 expression was positively related to deformities in the regression analyses, but failed to relate to the two components that explained most of the variation in principal component

analysis. Thus, the role of COX-1 in producing the AhR-mediated deformity phenotype, if any, requires clarification with further experiments.

The two COX-2 isoforms present in zebrafish are primarily involved in inflammatory responses (Tilley et al., 2001). While knockdown of COX-2a protects against TCDD-induced alterations in blood flow in the mesencephalic vein, it did not protect against blood flow changes induced in the trunk, indicating that COX-2a may have a more important role in the head region of developing zebrafish (Teraoka et al., 2009). In the current study, COX-2a was positively correlated with ventricular inner diameter, but similar to COX-1, failed to show a relation to the top components in principal components analysis. This apparent discrepancy may be at least partially explained by the observation that COX-2a expression was not increased after TCDD exposure when measured in whole larvae (Bugiak and Weber, 2009; Teraoka et al., 2009). Instead, small local changes in COX-2a, particularly in cardiac and vascular tissue, may be key in mediating AhR developmental toxicity. In contrast, the results of the current study indicate a protective effect of COX-2b to keep large blood vessel diameter within normal limits. The specific prostaglandin(s) produced by COX-2b may be essential for proper vascular development or regulation (Cha et al., 2005, 2006).

In conclusion, it was confirmed that BaP and TCDD induce different patterns of mRNA expression while neither Res nor ANF appear to be suitable for AhR antagonism *in vivo*. Concurrent exposure to BaP with ANF produces an additive toxic effect. Alterations in mRNA expression in response to AhR agonists and antagonists differ between 5 and 10 dpf with only CYP1A changes persisting to 10 dpf. Also, gene expression at 5 dpf, but not 10 dpf correlated with phenotype at 10 dpf. It was also shown that CYP1A, CYP1C1, CYP1C2, and COX-2a likely play a role in cardiac development while COX-2b may be important in vascular development of larval zebrafish. However, causality cannot be inferred from the data in our study. Further investigations using a variety of approaches should be carried out in order to ascertain the roles of these genes in AhR-induced toxicity in developing zebrafish. Although there were slight differences in types and severity of deformities caused by different AhR ligands, some similarities in phenotype were observed.

This suggests that there may be a unifying mechanism for the common effects that has yet to be determined.

5.0 Overall Discussion and Conclusions

This thesis aimed to elucidate the effect of AhR ligands on CYP and COX gene expression as well as the development and function of the cardiovascular system in zebrafish. After development of the necessary exposure, molecular and physiological methods needed for subsequent experiments, acute effects of AhR ligands in adult zebrafish were compared to developmental effects in larval zebrafish.

5.1 Tissue-Specific Versus Whole Animal Homogenate mRNA Expression

There have been many studies looking at AhR agonist toxicity in adult zebrafish but many of these relied on mortality or reproductive success as endpoints (Giesy et al., 2002; Heiden et al., 2005; King Heiden et al., 2009). There have been a few studies that looked at tissue-specific or whole larvae changes in mRNA expression of CYP enzymes (Godard et al., 2005; Wang et al., 2006), with a few more that have looked at changes in expression of different COX isoforms (Grosser et al., 2002; Cha et al., 2005; Teraoka et al., 2009). However, no study so far has examined AhR-mediated effects on both CYP and COX gene expression at the same time. Furthermore, most previous studies examining AhR-mediated changes in gene expression in adult fish have looked at hepatic tissue (Incardona et al., 2006; Jönsson et al., 2007a; Yu et al., 2008). The few that have looked at changes in cardiovascular gene expression have examined mesencephalic vein (Dong et al., 2002, 2004; Teraoka et al., 2009), rete mirabile (Schlezinger and Stegeman, 2000a; Garrick et al., 2005), or heart (Carney et al., 2006).

The larval experiments in this thesis identified changes in expression of multiple genes after exposure to AhR ligands, but these changes were based on a whole larval homogenate making it difficult to identify tissue-specific changes. Comparing to gene expression changes in adult tissues could provide clues to indicate which tissues are being

affected during larval development. For example, mRNA expression of all four CYP isoforms (1A, 1B1, 1C1, and 1C2) as well as COX-1 was increased in the mesenteric arteries of adult zebrafish after exposure to AhR agonists BaP and TCDD, while only CYP1A, CYP1C1, COX-1, and COX-2b were increased in hepatic tissue samples. Thus, increased larval CYP1C2 observed in the larval study may be due to changes in vascular gene expression. Conversely, increased larval COX-2b may be due to changes in hepatic gene expression. However, it is necessary to determine the ontogeny of the responsiveness of the receptor and gene battery in normal development to ensure that inducing these genes at an earlier time point does not have adverse effects on systems that are either unrelated to or secondarily affect the cardiovascular system. It would be very interesting to perform laser-assisted microdissection and subsequent rtrt-PCR on the developing liver and artery tissue in the zebrafish. This is one method that would allow more direct comparison of tissue-specific changes in larvae versus adult fish after AhR agonist exposure. Additionally, incorporating more genes into this analysis, such as NOS isoforms, would increase the scope and understanding of AhR ligand-mediated toxicity.

In contrast, CYP1A gene expression appeared to be increased after AhR agonist exposure in all tissues at all time points examined. Therefore, changes in CYP1A gene expression appear to lack tissue specificity. Despite this apparent lack of tissue specificity, CYP1A expression showed a strong correlation to a deformed cardiac phenotype in larvae. This correlation along with previous studies showing an increase in cardiac CYP1A expression after AhR ligand exposure (Carney et al., 2006; Jönsson et al., 2007a) supports a critical role for this gene in mediating AhR ligand toxicity.

5.2 Adult Effects Compared to Larval Effects

The concentrations and routes of exposure of the AhR ligands were quite different between the larval and adult exposures. The adults were exposed to relatively higher concentrations of the compounds, but for a shorter amount of time than the larvae.

Intraperitoneal injections in adults also ensured greater delivery of the AhR ligands to the fish compared to lower aqueous exposure of embryos, a method where less toxicant is likely to be bioavailable. Based on mortality responses in the current and previous studies, even though adult zebrafish received higher doses, larvae are much more sensitive to the effects of the AhR agonist exposure (Henry et al., 1997; Cantrell et al., 1998; Andreasen et al., 2002b; Bello et al., 2004; Incardona et al., 2004; Antkiewicz et al., 2005). Differences in bioavailability of the compounds used due to the routes of administration probably contributed to the different responses observed at the different life stages. With an ip injection, the compounds were likely metabolized in the liver before they reached effective levels systemically, whereas the aqueous exposure of the larvae allowed for gill uptake of the compounds, leading to rapid systemic distribution and effects before metabolism. There were no immediately observable physiological effects in adult fish after AhR ligand exposure despite changes in mRNA expression. However, histological analysis was not performed in the adult fish study to determine the extent of injury to the heart and vasculature. Measuring any cardiovascular physiological or morphological endpoint would have enabled the observed mRNA changes in adult fish to have greater biological meaning.

The mRNA expression changes seen in the adult tissues were of a greater magnitude than those seen in the larvae. Most notably, CYP1A expression in the adult hepatic and vascular tissues after exposure to TCDD was approximately 90- to 120-fold greater than the control group while in whole larvae CYP1A did not exceed a 16-fold difference above control groups at either time point examined. The CYP1A induction in larval fish has been reported to peak at 72 hours after exposure to AhR agonists (Prasch et al., 2003), but the larval fish in this thesis were analyzed 24 and 144 hours after the cessation of exposures. Possible explanations for lower larval expression changes after AhR agonist exposure could be due to either the earlier termination time-points or low exposure concentrations in the larval studies. Developing a method to expose the adult zebrafish in an aqueous manner similar to the larval exposure would enable a more direct comparison of mRNA expression between the two stages of development. Alternatively, the lower fold-induction of CYP1A in the larval studies in this thesis may instead be due to a developmental insensitivity to AhR-mediated pathways. The high mortality and

deformities observed after larval exposures argue against this latter possibility. A final possible explanation may be that CYP1A gene expression is not the primary mediator of AhR agonist toxicity. Further experiments examining adult responses to AhR agonists are needed to clarify these discrepancies.

5.3 Predicted Consequences of Developmental AhR Agonist Exposure

Many of the effects observed after developmental AhR agonist exposure would be detrimental to further larval development, making it unlikely that the fish would survive to adulthood. First, the gross deformities (i.e. spinal curvature, craniofacial deformities) would impede normal growth and development. The craniofacial deformities alone would hinder proper feeding, directly affecting growth and survival. The pericardial edema would put extensive pressure on the developing heart, potentially compounding heart malformations or at least promoting heart dysfunction. Furthermore, yolk sac edema is accompanied by failure to resorb yolk (Hill et al., 2004), ultimately leading to mortality of the larvae. Unlooped hearts would be lethal shortly after the time periods examined in this thesis (10 dpf), at least partially due to the decreased cardiac output reported in previous studies (King Heiden et al., 2009). However, even minor cardiac dysfunction would pose a problem later in the life of the fish as it may hamper the fish's ability to swim after prey or avoid predation. The findings of Heintz et al. (2000) support this idea because salmon exposed to low levels of PAHs in the laboratory, although grossly normal at the time of release into natural streams, returned to spawn in lower numbers than those that were reared in clean water.

The more subtle effects on the vasculature, such as increasing vessel diameter observed in this thesis, would also become detrimental to the fish as development progressed, potentially contributing to circulation failure. Dilated vessels could reduce blood pressure and particularly venous pressure sufficiently to impair ventricular filling.

Circulation failure has been reported to be inevitable after AhR agonist exposure (Henry et al., 1997), but this is likely dependent on higher exposure levels used in earlier mechanistic investigations (Walker and Peterson, 1994; Henry et al., 1997). At these high exposure levels, as circulation fails in peripheral vascular beds, the peripheral tissue begins to necrose and fins to deteriorate as a result of poor oxygen delivery. At lower, more environmentally relevant exposures, impaired perfusion may not significantly affect a fish under normal conditions. However, under stressful or high oxygen demand conditions such as hunting or predator avoidance, hypoxia caused by impaired perfusion may be severe enough to affect swim performance. While the concentrations of AhR ligands used in this thesis are lower than most previous studies, it remains unclear how these effects would have progressed if the larvae had been allowed to develop further. Therefore, examining lower concentrations of AhR agonist and growing the fish to adulthood is the next logical step in this line of investigation.

5.4 Overall Conclusions

This study has shown that BaP and TCDD, while having similar mechanisms, induce different mRNA expression patterns in both adult and larval zebrafish. Despite both compounds being known agonists of the AhR, there are likely other mechanisms of toxicity that are activated by these compounds. It was not possible to elucidate the exact mechanism of AhR-mediated toxicity from this study. Arteries from acutely exposed adult fish exhibited mRNA changes in a larger number of genes than the hepatic tissue, suggesting that arteries are more sensitive to AhR stimulation, particularly to ANF. Second, AhR ligand-induced mRNA changes precede physiological and morphological defects observed later in development. The mRNA changes observed in larvae were not nearly as dramatic as those seen in the isolated adult tissues, but it was important to be able to anchor the mRNA expression to specific phenotypes. Evidence from this thesis supports the hypothesis that CYP1A is a necessary mediator of AhR-induced toxicity. However, the

COX-2 isozymes may also play a key role in cardiovascular function and development of zebrafish.

5.5 Future Work

The results presented in this study are by no means conclusive. With the availability of the entire sequenced and annotated genome of the zebrafish, morpholino studies are the next logical choice for experiments. It would be naïve to assume that the genes analyzed here are the only ones involved in AhR-mediated toxicity. However, it is reasonable to assume that some of these genes are very important in the mechanism of AhR-mediated toxicity. Inhibiting these genes and analyzing the phenotypes that develop will provide more clues to the exact mechanism of AhR-mediated toxicity.

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